Binding of rat chylomicrons and their remnants to the hepatic low-density-lipoprotein receptor and its role in remnant removal

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Binding and uptake of rat chylomicrons of different metabolic stages by the hepatic low-density-lipoprotein (LDL) receptor were studied. Pure chylomicrons, characterized by apolipoprotein B-48 devoid of contaminating B-100, were labelled in their cholesteryl esters. Lymph chylomicrons and serum chylomicrons, enriched in apolipoprotein E and the C-apolipoproteins, bound poorly to rat hepatic membranes. In contrast, chylomicron remnants, containing the apolipoproteins B-48 and E, bound with high affinity. Specific binding of remnants was virtually completely competed for by LDL free of apolipoprotein E. In addition, in ligand blots both remnants and LDL associated with the same protein with an M_r characteristic of the LDL receptor. Uptake of remnants during a single pass through isolated perfused rat livers was decreased to about 50 % by an excess of LDL. It is concluded that rat chylomicron remnants are a ligand of the hepatic LDL receptor. The much higher affinity as compared with LDL is mediated by apolipoprotein E but not B-48, and is inhibited by the C-apolipoproteins. This explains why serum chylomicrons are not taken up by the liver, whereas remnants are rapidly removed from the circulation. Results from experiments *in vivo* suggest that the LDL receptor makes an important contribution to the hepatic uptake of remnants and may be the principal binding site of the liver responsible for remnant removal.

INTRODUCTION

The mechanism of the hepatic removal of chylomicron remnants is still controversial. Lipoproteins are taken up into cells by at least two pathways, i.e. LDL-receptor-mediated endocytosis and non-specific uptake, which is less well defined (Goldstein & Brown, 1982; Carew *et al.*, 1982; Dietschy & Spady, 1986). Hepatocytes are known to exhibit both. Because of these removal mechanisms and the accessibility to macromolecules owing to the fenestrated sinusoidal endothelium, the bulk of LDL, VLDL and chylomicron remnants is taken up by the liver (Nestel *et al.*, 1963; Carew *et al.*, 1982; Dietschy & Spady, 1986). The rate of uptake of lipoproteins will depend on the number and affinity of receptors. Previously the C-apolipoproteins were shown to inhibit premature uptake of chylomicron remnants by the liver, whereas apolipoprotein E enhances the removal (Windler *et al.*, 1980a; Windler & Havel, 1985). However, the lipoprotein receptors involved are still at issue. Recently much attention has been drawn to an apolipoprotein E-recognizing receptor distinct from the LDL receptor. The hypothesis of the existence of an additional receptor is based on the observation of apparently unimpaired chylomicron removal in familial hypercholesterolaemia and in Watanabe Heritable Hyperlipidaemic rabbits (Kita *et al.*, 1982). Indirect evidence has been obtained in studies showing incomplete competition of antibodies to the LDL receptor for binding of chylomicron remnants, and lack of influence of hormonal or metabolic variation of the LDL-receptor activity on the hepatic chylomicron-remnant removal (Hui *et al.*, 1981; Arbeeney & Rifci, 1984; Cooper *et al.*, 1987). Recently, two proteins from

liver have been shown to bind apolipoprotein E, one of which proved to be a mitochondrial ATPase, whereas the physiological role of the other is unclear (Beisiegel *et al.*, 1988). However, attempts to demonstrate directly or to isolate a chylomicron-remnant receptor have failed (Wade *et al.*, 1986). On the other hand, binding of remnants to specific binding sites of the liver has been shown (Carrella & Cooper, 1979). Yet the role of the hepatic LDL receptor cannot unequivocally be derived from available data (Carrella & Cooper, 1979; Cooper *et al.*, 1982, 1987; Hui *et al.*, 1984; Wade *et al.*, 1986). This is due to lack of documentation of the purity of the chylomicrons, characterized by apolipoprotein B-48 devoid of contaminating B-100, or of the lipoprotein receptor involved. However, on theoretical grounds chylomicron remnants as apolipoprotein E-containing particles should recognize the LDL receptor, as apolipoprotein E is a ligand of the LDL receptor with an affinity about 20-fold higher than that of apolipoprotein B-100 (Goldstein & Brown, 1982; Windler *et al.*, 1980b). Previous studies showed that more than 90 % of chylomicron remnants are taken up by hepatocytes sharing the intracellular pathway of VLDL and LDL (Chao *et al.*, 1981; Jones *et al.*, 1984).

Therefore it appeared necessary to evaluate the role of the LDL receptor in the hepatic remnant removal, by using pure chylomicron remnants containing only apolipoprotein B-48 but no B-100, and labelled in their core with [3 H]cholesteryl esters. A specific binding site on hepatic membranes was identified as the LDL receptor in competition studies and by ligand blots. The experiments were facilitated by a high activity of the specific binding site in this rat strain. Differences in the binding properties

Abbreviations used: LDL, low-density lipoproteins; VLDL, very-low-density lipoproteins; HDL, high-density lipoproteins.

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of lymph and serum chylomicrons and their remnants were characterized in relation to their contents of apolipoprotein E and the C-apolipoproteins. Evidence for a role of the LDL receptor in the hepatic uptake of remnants *in vivo* is presented.

MATERIALS AND METHODS

Animals and materials

Male Sprague-Dawley rats (Zentrale Versuchstieranstalt, Hanover, Germany) weighing 250–350 g were kept on normal laboratory chow and tap water. Rats were exsanguinated by aortic puncture under light diethyl ether anaesthesia, livers were removed and serum was obtained by low-speed centrifugation of the blood. Bovine serum albumin (fraction V), biotin hydrazide, sodium cyanoborohydride and Naphthol Blue Black were obtained from Sigma, St. Louis, MO, U.S.A. Sodium metaperiodate, H_2O_2 , 4-chloro-1-naphthol and Triton X-100 were purchased from E. Merck, Darmstadt, Germany. For preparative ultracentrifugation the rotors Ti 60, 50 and 50.3 (Beckman) were used, and for membrane assays the LP 42-rotor was used. 'Tris buffer' contained 50 mM-NaCl, 1 mM- CaCl_2 and 20 mM-Tris/HCl, pH 7.4. Radioactivity was counted in a gamma-counter (Packard), or in 5 ml of scintillation fluid (Rotizint; Roth Co., Karlsruhe, Germany) in a beta-counter (Packard) up to a counting error of less than 5%.

Preparation of lipoproteins

Human LDL were prepared by sequential ultracentrifugation in the density range of $d = 1.024$ – 1.050 by addition of KBr (Havel *et al.*, 1955). Normolipaemic human serum was centrifuged twice at the upper and lower density for $1.2 \times 10^8 g_{av}$ -min at 4°C and dialysed against several changes of Tris buffer. For labelling with ^{125}I (Na^{125}I ; 480–630 MBq/ μg of iodide, carrier-free; Amersham-Buchler, Braunschweig, Germany) the iodine monochloride method adapted for lipoproteins was applied (McFarlane, 1948). Unbound radioactivity was separated by chromatography on Sephadex G-25M (PD-10 columns; Pharmacia, Uppsala, Sweden) at room temperature. Labelled lipoproteins were eluted with Tris buffer at a flow rate of 1–1.5 ml/min. Over 98% of radioactivity was precipitable in 10% (v/v) trichloroacetic acid. The specific radioactivity ranged from 80000 to 200000 c.p.m./ μg of protein.

To obtain chylomicrons, the mesenteric lymph duct of rats was cannulated and lymph was collected on ice during intraduodenal infusion of 0.9% NaCl in 10% glucose as described by Imaizumi *et al.* (1978). Small chylomicrons were isolated by centrifuging lymph for $2.4 \times 10^7 g_{av}$ -min at 4°C and by re-centrifuging the supernatant under the same conditions. For radioactive labelling 310 kBq of $[1,2\text{-}^3\text{H}]$ cholesterol (Amersham-Buchler) complexed to albumin were added per h to the infusion, resulting in a specific radioactivity of 8–35 Bq/ μg of cholesteryl esters.

Rat serum free of triacylglycerol-rich lipoproteins was prepared by centrifugation of serum for $1.2 \times 10^8 g_{av}$ -min at $d = 1.019$ by the addition of $^2\text{H}_2\text{O}$ (Uvasol; $d = 1.11$; Merck) at 4°C . To obtain serum chylomicrons, the bottom fraction was incubated with lymph chylomicrons (1 mg of triacylglycerol per VLDL-free serum equivalent to 1 ml of the original serum) at 24°C for

1 h. Serum chylomicrons were re-isolated by centrifuging twice under the above conditions. To prepare chylomicron remnants, post-heparin plasma was obtained by bleeding rats 10 min after intravenous injection of 60 i.u. of heparin (Thrombophob; Nordmark, Hamburg, Germany)/kg body wt. and centrifuging the plasma as for VLDL-free serum. Lymph chylomicrons were incubated with the bottom fraction (1 mg of triacylglycerols per VLDL-free plasma equivalent to 1 ml of original plasma) at 37°C for 15 min. Remnants were isolated by centrifuging twice under the above conditions. Some $88.3 \pm 3.2\%$ of ^3H was in the cholesteryl esters ($n = 8$).

Preparation of rat liver membranes

Rat liver membranes were prepared essentially as described by Windler *et al.* (1980b). After removal livers were immediately chilled on ice, and the following procedures were carried out at 4°C . Livers were homogenized by five strokes, each of 10 s, with a Teflon pestle and centrifuged for $7500 g_{av}$ -min. The supernatant was centrifuged for $12000 g_{av}$ -min and the resulting supernatant re-centrifuged for $6 \times 10^6 g_{av}$ -min. The final pellet was diluted with Tris buffer to a final concentration of $10 \mu\text{g}$ of protein/ μl and stored in liquid N_2 . For preparation of Ca^{2+} -depleted membranes, Ca^{2+} -free Tris buffer containing 10 mM-EDTA was used. The final pellets were washed twice and centrifuged for $6 \times 10^6 g_{av}$ -min before storage in Ca^{2+} -free buffer without EDTA.

Membrane binding assay

Each sample of lipoproteins was incubated with $100 \mu\text{g}$ of membrane protein and, to allow subtraction of background radioactivity, without membrane in Tris buffer in a final volume of $100 \mu\text{l}$ at 37°C in polyallomer tubes (7 mm $\times$ 20 mm, Beckman). To each $75 \mu\text{l}$ of Tris buffer was added, and the samples were centrifuged for $3 \times 10^6 g_{av}$ -min at 4°C . The supernatant was replaced by $175 \mu\text{l}$ of fresh Tris buffer and re-centrifuged under the above conditions. The supernatant was removed, and the bottoms of the tubes containing the pellets were cut off and counted for radioactivity. The corresponding blanks were subtracted. When ^3H -labelled chylomicron remnants were incubated with liver membranes at 37°C , binding was maximal after 90 min. Therefore all succeeding incubations were carried out for this time. No binding was detected with rat erythrocytes or membranes prepared from erythrocytes.

Ligand blotting

Biotin was coupled to lipoproteins as described by Wade *et al.* (1985). Lipoproteins were oxidized with 4 mM-sodium metaperiodate in phosphate buffer, chromatographed on Sephadex G-25M as described above for radioiodination, and incubated with biotin hydrazide. Sodium cyanoborohydride (final concn. 1 mM) was added, and 12 h later reagents were removed by dialysis. Rat liver membranes (10 mg of protein) were solubilized in 1 ml of Tris buffer containing 1% Triton X-100 with gentle shaking at room temperature for 15 min. The soluble fraction was separated by ultracentrifugation for $6 \times 10^6 g_{av}$ -min at 4°C and subjected to 7%-polyacrylamide vertical-slab-gel electrophoresis in the presence of 0.1% SDS, run at 50 mA for 3–4 h at 5°C until the front marker (Bromophenol Blue) reached the bottom of the gel (Laemmli, 1970). M_r values were

Fig. 1. SDS/3%-polyacrylamide-gel electrophoretograms of apolipoproteins

Lanes: 1, human LDL; 2, rat VLDL ($d < 1.006$ for serum from fasted rats); 3, rat chylomicron remnants (prepared by hydrolysis of lymph chylomicrons in VLDL-free post-heparin plasma); 4, rat lymph chylomicrons. Approx. 400 μg of protein or 20 μg of LDL protein was applied to each gel.

Fig. 2. Isoelectric-focusing polyacrylamide-gel electrophoretograms (pH 3.5-7.0) of apolipoproteins

Lanes: 1, rat lymph chylomicrons; 2, rat serum chylomicrons (lymph chylomicrons that had been incubated in VLDL-free serum); 3, rat chylomicron remnants (prepared by hydrolysis of lymph chylomicrons in VLDL-free post-heparin plasma); 4 and 5, rat VLDL for comparison ($d < 1.006$ for serum from fasted or fed rats, respectively); 6, human LDL. Approx. 200 μg of protein or 400 μg of LDL protein was applied to each gel.

determined by using standard proteins (SDS-PAGE Molecular Weight Standards, high; Bio-Rad Laboratories, Richmond, CA, U.S.A.). Transfer to nitrocellulose membranes was performed at 200 mA for 16 h as described by Burnette (1981). Standard proteins were stained with Naphthol Blue Black as described by Beisiegel *et al.* (1982). Receptor proteins were detected by using biotinylated lipoproteins as described by Wade *et al.* (1985). Incubation was carried out at 37 °C for 90 min, since by this time binding of lipoproteins to membranes was shown to have reached its maximum. In some experiments the incubation buffer contained no Ca^{2+} , but 10 mM-EDTA. Concentrations of biotinylated lipoproteins varied between 10 and 100 μg of protein/ml, with the best detection at 40 μg /ml by streptavidin/biotinylated-peroxidase complex.

Uptake of lipoproteins by perfused rat livers

Removal of [^3H]cholesteryl esters in chylomicron remnants by perfused livers was measured by a single-pass technique essentially as described by Windler *et al.* (1980b). Isolated livers were perfused with 20 ml of Krebs-Henseleit buffer, pH 7.4, containing 20% (w/v) rat erythrocytes in a recycling system for 30 min. After exchange with the same volume of fresh perfusate, lipoproteins (350 μl) were injected into the tubing leading to the liver over 10 s, and the perfusate was collected on the venous side. After flushing with 20 ml of buffer, the livers were stored at -20 °C. Three pieces (each about 100 mg) were dissolved in 0.5 ml of Protosol (New England Nuclear, Boston, MA, U.S.A.). The liver specimens and three 1 ml samples of each perfusate were

counted for radioactivity and corrected for quench by an internal standard.

Analyses

Protein was determined as described by Lowry *et al.* (1951) as modified for lipoproteins, with bovine albumin as standard (Sata *et al.*, 1972). Lipids were determined by standard procedures with reagents from Boehringer, Mannheim, Germany (Wahlefeld, 1974; Stähler *et al.*, 1977). Radioactivity in lipids was determined after extraction and separation by t.l.c. (Bligh & Dyer, 1959; Fielding, 1979). Polyacrylamide-gel electrophoresis of apolipoproteins involved isoelectric focusing in the absence of reducing agents and in SDS as modified for apolipoprotein B (Weber & Osborn, 1969; Pagnan *et al.*, 1977; Kane *et al.*, 1980). Electron microscopy was performed on lipoproteins negatively stained with 2% (w/v) phosphotungstic acid, with a Philips EM 300 instrument. For multiple experiments, results are given as means $\pm$ S.D. for n experiments.

RESULTS

Characterization of lipoproteins

Lipoproteins were analysed by polyacrylamide-gel electrophoresis. Electrophoretograms, in the presence of SDS, of human LDL showed one major band with the mobility characteristic of apolipoprotein B-100 and the equivalent band of rat VLDL (Fig. 1) (Kane, 1983). Below this an additional faint band was visible, suggesting the presence of apolipoprotein B-96 (Kane, 1983). Chylomicrons isolated from rat mesenteric lymph yielded

Table 1. Distribution of triacylglycerols and cholesterol in lipoproteinsResults are means $\pm$ S.D. ($n = 6$)

Lipoproteins	Content (% by wt.)		
	Triacylglycerols	Unesterified cholesterol	Cholesteryl esters
LDL	8.6 $\pm$ 1.7	15.6 $\pm$ 2.0	75.8 $\pm$ 1.8
Lymph small chylomicrons	92.1 $\pm$ 0.8	4.4 $\pm$ 1.9	3.4 $\pm$ 1.6
Serum small chylomicrons	90.9 $\pm$ 1.9	3.8 $\pm$ 1.4	5.3 $\pm$ 1.3
Small chylomicron remnants	82.1 $\pm$ 3.7	6.1 $\pm$ 2.1	11.8 $\pm$ 2.4

a band in the position of apolipoprotein B-48, but none in that of apolipoprotein B-100 (Fig. 1). This was also true for remnants produced from these chylomicrons by incubation in VLDL-free post-heparin plasma.

Isoelectric-focusing electrophoretograms of LDL did not stain for any of the soluble apolipoproteins (Fig. 2). No apolipoprotein E was detected by specific rocket immunoelectrophoresis with a sensitivity of 0.5 μ g/ml, equivalent to less than 0.002% of LDL protein (Laurell, 1966). Lymph chylomicrons yielded the apolipoproteins A-I, C-II, C-III-0 and C-III-3, identified as described previously (Fig. 2) (Windler *et al.*, 1980a). In the region of the apolipoproteins E and A-IV, which overlap in isoelectric-focusing gels, the two bands characteristic of apolipoprotein A-IV prevailed (Windler *et al.*, 1980a). In contrast, lymph chylomicrons that had been incubated in VLDL-free serum (serum chylomicrons) predominantly yielded the pattern of apolipoprotein E. The bands of apolipoprotein A-I and A-IV were diminished, whereas those of the C-apolipoproteins were increased (Fig. 2). In the case of chylomicron remnants, the isoforms of apolipoprotein E were visible as the only soluble proteins

(Fig. 2). Provided that there was an unchanged content of cholesteryl esters, an average of 74.3% of triacylglycerols in the parent chylomicrons were hydrolysed during remnant formation. The distribution of cholesterol and triacylglycerols in lipoproteins is given in Table 1. Ratios of protein to cholesteryl esters were 0.7 ± 0.2 , 2.6 ± 1.0 and 1.1 ± 0.5 (w/w) for LDL, lymph chylomicrons and remnants respectively. Various preparations of lipoproteins examined by electron microscopy appeared spherical and intact.

Binding of lipoproteins to rat liver membranes

At increasing concentrations, ^3H -labelled remnants bound to liver membranes in a saturable manner (Figs. 3 and 4). Subtraction of binding in the presence of excess unlabelled remnants gave the specific binding. Saturation occurred at a concentration of about 100 μ g of cholesteryl ester/ml, corresponding to approx. 40 ng of cholesteryl ester bound/ μ g of membrane protein. Comparable results were obtained in experiments with freshly prepared membranes and whole-liver homogenate subjected only to one centrifugation for 500 g_{av} -min at 4 °C (Fig. 3). Binding activity was preserved in membranes stored in liquid N_2 for several weeks.

To test for Ca^{2+} -dependence of binding of ^3H -labelled chylomicron remnants, liver membranes were prepared in the absence of Ca^{2+} ions. Binding to these membranes in Ca^{2+} -free medium was low and comparable with that to control membranes in the presence of excess remnants or LDL (Table 2). Specific binding could be restored by addition of Ca^{2+} , which again was abolished by excess LDL. Comparable results were obtained for LDL (results not shown).

When ^3H -labelled chylomicrons were incubated with liver membranes, binding was far less than for remnants at the same concentration of cholesteryl esters (Fig. 4). Virtually all binding was specific. For ^3H -labelled lymph chylomicrons it was even slightly lower than for ^3H -labelled serum chylomicrons.

The plot of total radioactivity of ^{125}I -LDL bound to liver membranes was curvilinear (Fig. 5). Specific binding, deduced by subtraction of the binding in the presence of

Fig. 3. Binding of ^3H -labelled chylomicron remnants to rat liver membranes at 37 °C in the absence and presence of a 20-, 16- and 8-fold excess of unlabelled remnants at 30, 100 and 200 μ g of ^3H cholesteryl esters/ml respectively.

(a) Freshly prepared liver homogenate, centrifuged once for 500 g_{av} -min at 4 °C. (b) Membranes prepared from liver homogenate which was centrifuged at 8000 g ; the supernatant was re-centrifuged at 100 000 g ; the pellet containing the membranes was stored in liquid N_2 . Values are means of triplicate assays.

Fig. 4. Binding of ^3H -labelled chylomicron remnants (prepared by hydrolysis of lymph chylomicrons in VLDL-free plasma), ^3H -labelled serum chylomicrons (lymph chylomicrons that had been incubated in VLDL-free serum) and ^3H -labelled lymph chylomicrons to rat liver membranes at 37°C in the absence and presence of a 10-fold excess of the respective unlabelled lipoprotein or human LDL (3 mg of cholesteryl ester/ml)

Values are means $\pm$ s.d. for three experiments in triplicate assays.

excess unlabelled LDL, was saturated at about $300\ \mu\text{g}$ of LDL-cholesteryl esters/ml, with a maximal binding of about 6 ng of LDL-cholesteryl esters/ μg of membrane protein.

Competition of remnants and LDL for binding sites of rat liver

Competition studies were performed at the concentration of labelled lipoproteins where specific binding began to be saturated. Binding of ^{125}I -LDL was increasingly competed for by rising concentrations of unlabelled LDL, until a maximum of about 55% suppression was attained at a 14-fold excess of unlabelled LDL (Fig. 6). Displacement of the binding of ^3H -labelled remnants was almost complete at a 5-fold excess of unlabelled remnants and was 91% at a 10-fold excess (Fig. 6). Bound ^3H -labelled remnants could also be displaced by LDL, up to a maximum of 90% (Fig. 6); 50% displacement was achieved at a ratio of about 1:30 of ^3H -labelled remnant to LDL-cholesteryl ester. Effective suppression of the specific binding by LDL was possible at low as well as at high concentrations of ^3H -

Table 2. Binding of ^3H -labelled chylomicron remnants to rat liver membranes in the presence and absence of Ca^{2+}

Rat liver membranes were prepared in Tris buffer either containing 1 mM- Ca^{2+} or containing no Ca^{2+} but 10 mM-EDTA, which was finally removed by two washings. Results are means $\pm$ s.d. ($n = 3$).

	Binding (ng of cholesteryl esters/ μg of membrane protein)
Membranes prepared in the presence of Ca^{2+}	
^3H -labelled remnants (120 μg of cholesteryl esters/ml)	45.5 ± 8.6
+ remnants (1.2 mg of cholesteryl esters/ml)	6.2 ± 3.5
+ LDL (3 mg of cholesteryl esters/ml)	8.1 ± 2.8
Membranes prepared in the absence of Ca^{2+}	
^3H -labelled remnants (120 μg of cholesteryl esters/ml)	15.9 ± 0.4
+ Ca^{2+} (1 mM)	57.8 ± 2.4
+ Ca^{2+} , + LDL (1 mM) (3 mg of cholesteryl esters/ml)	15.5 ± 1.4

Fig. 5. Binding of human ^{125}I -LDL to rat liver membranes at 37°C in the absence and presence of excess unlabelled LDL (3 mg of cholesteryl ester/ml)

Values are means $\pm$ s.d. for three experiments in triplicate assays.

labelled remnants (Fig. 4). For competition with LDL, 3 mg of cholesteryl ester/ml was used at all concentrations of remnants, to ensure saturation of the LDL receptor (Fig. 4).

Binding experiments also utilized membrane proteins that had been separated by SDS/polyacrylamide-gel

Fig. 6. Competition of increasing concentrations of unlabelled lipoproteins with labelled lipoproteins

(a) Competition of unlabelled human LDL with ^{125}I -LDL ($105\text{ }\mu\text{g}$ of cholesteryl ester/ml). (b) Competition of unlabelled chylomicron remnants with ^3H -labelled chylomicron remnants ($120\text{ }\mu\text{g}$ of cholesteryl ester/ml). Values are means $\pm$ S.D. for three experiments in triplicate assays. (c) Competition of human LDL with ^3H -labelled chylomicron remnants ($120\text{ }\mu\text{g}$ of cholesteryl ester/ml). Data from individual experiments in triplicate assays are shown. Ratios on the abscissa are in terms of cholesteryl ester.

electrophoresis and blotted on nitrocellulose (Fig. 7). After incubation with either biotinylated LDL or remnants, a single band was observed at the same position for both lipoproteins, corresponding to a molecular mass of 130–135 kDa. The same result was obtained with proteins of whole liver homogenate and the 100000 g supernatant. No additional binding site

Fig. 7. Ligand blots of biotinylated human LDL and rat chylomicron remnants to rat liver membranes

Membrane proteins were separated by SDS/polyacrylamide-gel electrophoresis, blotted on nitrocellulose and incubated with biotinylated lipoproteins ($20\text{ }\mu\text{g}$ of protein/ml). Results of three experiments are shown.

Fig. 8. Removal of ^3H -labelled chylomicron remnants in the absence and presence of excess human LDL by isolated perfused rat livers

Uptake of $60\text{ }\mu\text{g}$ of [^3H]cholesteryl esters was measured by a single-pass technique. Excess of LDL is given as the ratio of cholesteryl esters in LDL to cholesteryl esters in remnants. Bars indicate means of the individual experiments, determined in triplicate assays.

was observed. Binding of LDL or remnants in the presence of unlabelled LDL (1.5 mg of protein/ml) resulted in disappearance of this band. Also, ligand blots in the presence of 10 mM-EDTA and in the absence of Ca^{2+} showed no, or markedly decreased, binding of remnants (results not shown).

After a single pass of ^3H -labelled remnants through isolated perfused rat livers, a mean of 46 % of the dose

of 60 μg of [^3H]cholesteryl esters was recovered in the effluent perfusate (Fig. 8). When both ^3H -labelled remnants and excess unlabelled LDL at a ratio of 1:350 of cholesteryl ester were perfused through livers, an average of 74% of [^3H]cholesteryl esters was recovered in the perfusate. A greater excess of LDL at a ratio of 1:450 had no additional effect. To exclude non-specific absorption of lipoproteins to the tubing, in six experiments recovery of ^3H was determined, and was $99.7 \pm 5.7\%$ in perfusate plus liver.

DISCUSSION

This investigation demonstrates that chylomicron remnants and LDL share the hepatic LDL receptor as a common binding site and suggests that this pathway is of importance for the removal of remnants *in vivo*.

A prerequisite for this study was the isolation of chylomicron remnants and LDL in a pure form, in order to be able to distinguish between binding to the LDL receptor and a possible apolipoprotein E receptor. Although lipoproteins containing apolipoprotein B-100 but no apolipoprotein E are supposed to bind exclusively to the LDL receptor, apolipoprotein E-containing particles have been suggested to bind to an additional receptor (Kita *et al.*, 1982; Hui *et al.*, 1981). To minimize the risk of contamination with apolipoprotein E, LDL were isolated within the narrow density range of 1.024–1.050 g/ml. Human serum was used, as significant amounts of LDL free of apolipoprotein E cannot be prepared from rat plasma (Windler *et al.*, 1980b). Though binding to the apolipoprotein E receptor of LDL, even from a broader density range, has not been reported, the absence of apolipoprotein E will strengthen the validity of the results of this study. On the other hand, chylomicrons and remnants had to be obtained that contain only apolipoprotein B-48 but no B-100, in order not to mistake binding of apolipoprotein B-100-containing lipoproteins as VLDL to the LDL receptor for binding of chylomicrons. This requirement has been met by using lymph from the mesenteric duct, in contrast with the thoracic duct which contains lymph of hepatic origin, and by preparing serum chylomicrons or remnants in VLDL-free serum or plasma instead of using functionally eviscerated rats (Carrella & Cooper, 1979; Tamai *et al.*, 1983; Wade *et al.* 1986). Additionally, chylomicrons were biologically labelled in their cholesteryl esters, a non-exchanging label in the core, in order to minimize alteration resulting from labelling procedures *in vitro* and to circumvent errors owing to redistribution of radioiodinated soluble apolipoproteins. The fact that binding of ^3H -labelled chylomicron remnants is completely displaced by unlabelled remnants or LDL excludes significant redistribution of labelled components in our assay. Yet comparable results were also obtained with chylomicron remnants labelled with ^{125}I (results not shown).

In this investigation the widely used crude preparation of rat liver membranes originally described by Kovanen *et al.* (1979) was chosen. This approach minimizes the risk of losing binding activity during the preparation procedure or owing to selection of subfractions of the plasma membrane. The presence of intracellular membranes requires demonstration of binding to a site known to be located on the cell surface as the LDL receptor.

In order to perform reliable competition studies, it was

necessary to show specific binding of human LDL to hepatic membranes from normal rats, which is in agreement with recent reports (Roach & Noël, 1985; Brissette & Noël, 1986; Cooper *et al.*, 1987), since in previous studies only indirect evidence could be obtained (Windler *et al.*, 1980b; Kovanen *et al.*, 1979). In this investigation maximal specific binding was similar to what has previously been found in oestrogen-treated rats (Kovanen *et al.*, 1979). This may be due to differences in the animal strain, but also to age, since we found the same binding to liver membranes from 1-week-old and 2–3-month-old rats used for this research, in contrast with greatly decreased binding with 6-month-old rats (E. Windler & J. Greeve, unpublished work). Ca^{2+} -dependence of the LDL receptor has long been recognized (Windler *et al.*, 1980b). In this study, specific binding of LDL and chylomicron remnants was abolished by preparation of membranes in Ca^{2+} -free medium, but was restored by addition of Ca^{2+} . This finding suggests that the membrane assay represents specific binding to the LDL receptor.

This is further supported by the virtually complete displacement of remnants by LDL. At all concentrations tested, even at low concentrations of chylomicron remnants, LDL efficiently suppressed binding of remnants to the same extent as for unlabelled remnants. To ensure maximal competition, at all concentrations of remnants an amount of LDL well above the saturating concentration was used, as determined from the binding curve (Figs. 4 and 5). In such studies a constant ratio of ligand to competitor may lead to insufficient concentrations of competitor, with incomplete suppression at low concentrations of ligand. This is especially important in experiments with two ligands that differ substantially in their affinity. To achieve higher sensitivity for competition, a concentration of ligand close to saturation was used. Moreover, this allowed estimation of the portion of remnant-binding sites shared by LDL. On the basis of cholesteryl esters, a 30-fold excess of LDL was necessary to displace 50% of remnants, i.e. remnants exhibit a manyfold higher affinity to the LDL receptor than did LDL itself, of the order of what has been found for the affinity of apolipoprotein E compared with B-100 (Goldstein & Brown, 1982; Windler *et al.*, 1980b).

Binding of LDL and chylomicron remnants to the same receptor was confirmed by a second approach. In ligand blots chylomicron remnants and LDL bound to a protein of the same apparent relative molecular mass reported for the hepatic LDL receptor of the rat (Wade *et al.*, 1986; Cooper *et al.*, 1987). There was no difference whether human LDL or rat LDL isolated as a pure fraction by zonal ultracentrifugation was used (W. Daerr, E. Windler & J. Greeve, unpublished work).

The results of the binding studies *in vitro* are supported by the experiments with perfused livers. The competition of remnants by LDL *in vivo* strongly suggests that the binding of remnants to the hepatic LDL receptor is not only an effect observed *in vitro*, but it also occurs *in vivo* and contributes to the removal of chylomicron remnants by the liver. The fact that 50% uptake of remnants is not suppressed is compatible with the existence of additional removal mechanisms. Yet this does not necessarily implicate receptor-dependent uptake, since non-receptor-mediated removal mechanisms, which have been demonstrated in the liver, may efficiently act on

chylomicron remnants (Carew *et al.*, 1982). Turnover data indicate that large amounts of LDL are internalized via this pathway under normal conditions, and especially in states of decreased or absent LDL receptors, as in patients with homozygous familial hypercholesterolaemia (Goldstein & Brown, 1982). The efficacy of this pathway to take up chylomicron remnants has never been determined. Yet this investigation probably provides a minimal estimate of the contribution of the LDL receptor to remnant removal, since *in vivo* limited redistribution of LDL in the spaces of Disse may lead to incomplete saturation of the LDL receptor. Small numbers of unoccupied receptors may be sufficient for remnant removal, because of their high affinity.

In contrast with the LDL receptor, it has not been possible to demonstrate an additional remnant receptor directly by binding assays using hepatic membranes of the rat. Research designed to demonstrate an apolipoprotein E receptor by ligand blotting has been unsuccessful, despite a variety of experimental conditions (Wade *et al.*, 1986). Proteins purified by apolipoprotein E affinity chromatography from liver cells, the major of which is identical with a mitochondrial ATPase, were shown to bind apolipoprotein E-containing lipoproteins, yet whether they play a role in lipoprotein metabolism needs to be determined (Beisiegel *et al.*, 1988). Even freshly prepared liver homogenate did not yield evidence for a binding site in addition to the LDL receptor in our experiments, whereas the activity of the LDL receptor was preserved during membrane preparation and storage. In any case, the observed decrease in hepatic remnant uptake by competition with LDL in liver perfusions strongly argues for a role of the LDL receptor in remnant removal. The high affinity of remnants for the LDL receptor may be responsible for the fast turnover of remnant particles and the lack of impairment of the rate of hepatic uptake even in states of lower LDL-receptor number, as in Watanabe Heritable Hyperlipidaemic rabbits, which possess only about 5% of the normal LDL-receptor activity (Kita *et al.*, 1982).

Serum chylomicrons bound with much lower affinity to the LDL receptor than did their remnants. Both contain apolipoprotein E, but differ in that serum chylomicrons are rich in C-apolipoproteins. In earlier work the C-apolipoproteins have been shown to inhibit the uptake of chylomicrons and their remnants by perfused livers, though the underlying mechanism was unknown (Windler *et al.*, 1980a; Windler & Havel, 1985). This investigation suggests that the C-apolipoproteins specifically affect the affinity of apolipoprotein E for its receptor site. This is in agreement with results obtained with VLDL (Windler *et al.*, 1980b). Lymph chylomicrons showed even less affinity to the LDL receptor than did serum chylomicrons. This is best explained by their low content of apolipoprotein E in the presence of some C-apolipoprotein, and is in accordance with experiments with perfused rat livers or dog hepatic receptors, indicating that, in contrast with apolipoprotein B-100, B-48 is no ligand of the LDL receptor (Hui *et al.*, 1984; Borensztajn *et al.*, 1982). The above findings allow explanation of earlier observations with chylomicrons (Windler *et al.*, 1980a; Windler & Havel, 1985). Their change in half-life during the intravascular metabolism can be attributed to alterations of their affinity to hepatic receptors, owing to variations in the apolipoprotein composition.

In conclusion, this investigation characterizes the LDL receptor as a binding site for chylomicron remnants. The affinity appears to be determined by the content of apolipoprotein E and the C-apolipoproteins. The results of experiments with isolated perfused livers point to an important role of the LDL receptor in the removal of chylomicron remnants *in vivo*. The existence of an additional receptor appears questionable, and the significance of non-receptor-mediated uptake remains to be determined.

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