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Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoprotein E-enriched lipoproteins

(β-very low density lipoproteins/chylomicrons/familial hypercholesterolemia/cell-surface receptors/lysosomal hydrolysis)

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ABSTRACT **Low density lipoprotein receptor-related protein (LRP) is a recently described cell-surface protein of 4544 amino acids that contains reiterated sequences found in the 839-amino acid receptor for low density lipoprotein (LDL). In the current studies, we purified LRP from rat liver, prepared polyclonal antibodies that recognize the extracellular domain, and demonstrated an immunoreactive protein of ≈600 kDa in human fibroblasts. The function of this LRP was studied in mutant human fibroblasts that do not produce LDL receptors. The mutant cells were incubated with β-migrating very low density lipoprotein (β-VLDL) that was isolated from cholesterol-fed rabbits and artificially enriched with apoprotein (apo) E by incubation *in vitro* with human apo E produced in a bacterial expression system. The apo E-enriched β-VLDL, but not unincubated β-VLDL, stimulated incorporation of [^{14}C]-oleate into cholesteryl [^{14}C]oleate 20- to 40-fold in the mutant cells. This stimulation was blocked by chloroquine, suggesting that such stimulation resulted from receptor-mediated uptake and lysosomal hydrolysis of the cholesteryl esters in apo E-enriched β-VLDL. Stimulation of cholesterol esterification was blocked by the antibody against LRP, but not by an antibody against the LDL receptor. Unlike the LDL receptor, the amount of LRP was not reduced when cells were incubated with oxygenated sterols. We conclude that LRP can mediate the cellular uptake and lysosomal hydrolysis of cholesteryl esters contained in lipoproteins that are enriched in apo E.**

Lipoproteins that contain apoproteins (apo) B or E are removed from plasma by binding to high-affinity receptors on the liver and extrahepatic tissues. At least two receptors play a role (1). The low density lipoprotein (LDL) receptor binds LDL, which contains only apo B-100. It also binds apo E-containing remnants of chylomicrons and very low density lipoproteins (VLDL) that are produced by lipoprotein lipase. Approximately 70% of LDL receptors are located in the liver, and the rest are found in adrenal gland, ovary, and other extrahepatic tissues (1).

The existence of a second receptor was suggested by observations in patients with homozygous familial hypercholesterolemia (FH) and WHHL rabbits in whom LDL receptors are genetically defective (2). Although VLDL remnants and LDL accumulate in blood of affected individuals, chylomicron remnants do not (3). WHHL rabbits clear chylomicron remnants normally (4), and they also rapidly clear an appreciable fraction of large VLDL particles from blood (5). These observations suggest that the second receptor is specific for apo E-containing chylomicron remnants and large VLDL. Repeated attempts to define this receptor biochemically have been unsuccessful.

A major advance has recently come from the work of Herz *et al.* (6), who used homology cloning to isolate a cDNA for an LDL receptor-related protein (LRP). The LRP resembles the LDL receptor in containing two types of cysteine-rich repeats, one related to complement proteins and the other related to epidermal growth factor. The LDL receptor contains 7 of the former and 3 of the latter repeats; LRP contains 31 and 22 repeats, respectively. The entire LRP consists of 4544 amino acids as compared with 839 amino acids for the mature LDL receptor. Like the LDL receptor, the LRP binds calcium. The LRP is present on the surface of cultured human hepatoma cells (HepG2 cells), and LRP mRNA is found in many organs, including the liver, lung, and brain (6). The cytoplasmic tail of LRP is twice as long as the cytoplasmic tail of LDL receptor, and the LRP cytoplasmic tail contains two copies of a tyrosine-containing sequence necessary for internalization of the LDL receptor in coated pits (7). Herz *et al.* (6) postulated that the LRP might function as a lipoprotein receptor and that it may also play a role in growth, owing to the growth factor-like sequences. Beisiegel *et al.* showed that LRP in HepG2 cells or isolated liver membranes could be chemically crosslinked to apo E (U. Beisiegel, W. Weber, G. Ihrke, J.H., and K. Stanley, unpublished work). LRP has not been purified, and the only available antibodies are antipeptide antibodies directed at the COOH-terminal cytoplasmic tail (6).

In the current studies we purified the LRP from rat liver to homogeneity and prepared a polyclonal antibody that reacts with the external domain. We used mutant human fibroblasts that lack the LDL receptor to show that LRP mediates the uptake of apo E-enriched lipoproteins in a way that is inhibited by the anti-LRP antibody. These studies indicate that the LRP may, indeed, function as a receptor for apo E-enriched lipoproteins.

MATERIALS AND METHODS

Purification of LRP. LRP was purified from rat livers by a modification of the method used for purification of the LDL receptor (8). Triton X-100-solubilized membrane extracts were bound to DEAE-cellulose and eluted with a 500-ml linear gradient from 0 to 1 M NaCl in a buffer containing 50 mM Tris maleate (pH 6.0), 2 mM $CaCl_2$, 1% (vol/vol) Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. Fractions containing LRP were pooled, adjusted to pH 7.5 with 2 M Tris, and centrifuged at 100,000 × *g* for 45 min at 4°C. The supernatant was applied to a column containing anti-LRP monoclonal IgG-11H4 (see below) coupled to cyanogen bromide-activated Sepharose 4B. This column was washed with 250 ml of 50 mM Tris hydrochloride (pH 7.5), 2 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100, followed by 6 ml of 50 mM NH_4HCO_3. LRP was eluted with 10 ml of 0.1 M NH_4OH, frozen in liquid N_2, and lyophilized.

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Abbreviations: apo, apoprotein; FH, familial hypercholesterolemia; LDL, low density lipoprotein; LRP, LDL receptor-related protein; VLDL, very low density lipoprotein; β-VLDL, β-migrating VLDL.

For experiments LRP was resuspended in 50 mM Tris hydrochloride (pH 8.0) and 2 mM $CaCl_2$.

Antibodies. A rabbit polyclonal antibody against rat LRP was prepared as described (9) except that animals received 30 μg of LRP per injection. A mouse monoclonal antibody (IgG-11H4) against a synthetic peptide corresponding to the 13 COOH-terminal amino acids of human LRP (6) was prepared as described (10). Polyclonal and monoclonal antibodies against the bovine LDL receptor were prepared as described (11). IgG fractions were obtained with Protein A–Sepharose chromatography (9).

Cells. Diploid human skin fibroblasts were grown in monolayer at 37°C in 5% CO_2 (12). FH 808 is a French Canadian who is homozygous for a >10-kilobase (kb) deletion that removes the promoter and first exon of the LDL receptor gene (13).

Lipoproteins. β-Migrating VLDL (β-VLDL) was prepared from plasma of cholesterol-fed rabbits as described (14), except that it was centrifuged twice at ρ 1.006 g/ml. Recombinant human apo E (isoform E-3), obtained from *Escherichia coli* (batch H5-29) (15), was provided by Tikva Vogel (Biotechnology General, Rehovot, Israel).

Blot Hybridization of RNA. Total RNA was isolated from human fibroblasts with guanidine hydrochloride (16). Blot hybridization of LRP mRNA was performed as described (17) with two single-stranded ^{32}P-labeled rabbit cDNA probes (corresponding to nucleotides 287–3142 and 509–3280 of human LRP cDNA). Rabbit LRP cDNA was isolated by screening a rabbit liver cDNA library with the human cDNA. For analysis of LDL receptor mRNA (17), a single-stranded ^{32}P-labeled probe corresponding to nucleotides 2150–2548 of the human LDL receptor cDNA (18) was used.

Cholesteryl Esterification Assay. About 3–4 × 10^4 fibroblasts were seeded into 60-mm Petri dishes and grown as described (12), except that they were switched to lipoprotein-deficient medium on day 6 rather than day 5. On day 7 each monolayer received 2 ml of Dulbecco's modified Eagle medium (without glutamine) containing bovine serum albumin at 2 mg/ml (medium A) and the indicated additions. β-VLDL and apo E were preincubated together for 1 hr at 37°C in 0.3–0.5 ml of medium A before addition to the culture medium. After the indicated time the cells were pulse-labeled for 2 hr at 37°C with 0.2 mM [^{14}C]oleate bound to albumin (12) at a specific activity of 8760–10,112 dpm/nmol and harvested for measurement of cholesteryl [^{14}C]oleate and [^{14}C]triglycerides (12).

RESULTS

Fig. 1 (*Left*) shows silver-stained gels of a crude rat liver membrane extract (lane 1), pooled DEAE-cellulose fractions containing LRP (lane 2), and the purified LRP after passage over an affinity column containing a monoclonal antipeptide antibody directed against the COOH-terminal tail (lane 3). The purified protein migrated with an apparent M_r of ≈600,000, as previously observed (6). A rabbit polyclonal antibody raised against the purified rat LRP stained the 600-kDa protein in extracts from normal human fibroblasts (lane 5). The antibody also stained an 85-kDa protein that is a proteolytic degradation product of LRP (6).

Normal human fibroblasts contain an mRNA of about 15 kb that hybridizes with a ^{32}P-labeled rabbit LRP cDNA (Fig. 2, lane 1). A similar mRNA was present in fibroblasts from FH 808 (lane 2). This subject is homozygous for the French Canadian mutation, a deletion of >10 kb that removes the first exon and the promoter of the LDL receptor gene and prevents all transcription (13). The mRNA for the LDL receptor was present in normal fibroblasts (lane 3), but not in FH 808 fibroblasts (lane 4).

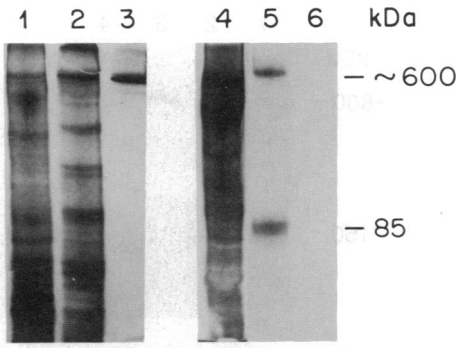

FIG. 1. (*Left*) SDS/PAGE of sequential fractions in the purification of rat LRP. Aliquots of membrane extracts (20 μg, lane 1), pooled DEAE-cellulose fractions (5 μg, lane 2), and affinity-purified LRP (0.5 μg, lane 3) were subjected to electrophoresis on a 5% gel and stained with silver. Protein concentration was determined with BCA* protein assay reagent (Pierce). (*Right*) Immunoblot analysis of LRP from normal human fibroblasts. Cells were solubilized with Nonidet P-40, and the nuclei were removed by centrifugation (6). Proteins (15 μg/lane) were subjected to 5% SDS/PAGE and either stained with silver (lane 4) or transferred to nitrocellulose (lanes 5 and 6). The strips were incubated with rabbit anti-rat LRP IgG at 5 μg/ml (lane 5) or preimmune IgG (lane 6) at 5 μg/ml, followed by ^{125}I-labeled goat anti-rabbit IgG (375 ng/ml; 8000 cpm/ng). The strips were exposed to Kodak XRP-1 film for 13 hr at 20°C. The gel was calibrated with apo B-100 (512 kDa), myosin (200 kDa), phosphorylase (92 kDa), and bovine serum albumin (68 kDa).

Normal and FH 808 fibroblasts incorporated [^{35}S]cysteine into a 600-kDa protein that was precipitated with the polyclonal anti-LRP antibody (Fig. 3*A*, lanes 1 and 3). This protein was not decreased when cells were grown with 25-hydroxycholesterol (lanes 2 and 4). The normal fibroblasts also produced a protein with an apparent molecular mass of 160 kDa that was precipitated with the anti-LDL receptor antibody (Fig. 3*B*, lane 1). The amount of LDL receptor was markedly reduced in the 25-hydroxycholesterol-treated cells (lane 2). In contrast, FH 808 fibroblasts failed to produce any LDL receptors (lanes 3 and 4).

The availability of the FH 808 cells provided the opportunity to study LRP-mediated lipoprotein uptake in cells lacking LDL receptors. A sensitive assay for such uptake is the stimulation of [^{14}C]oleate incorporation into cholesteryl [^{14}C]oleate, an event that requires hydrolysis of the cholesteryl esters in lysosomes (12). As a potential ligand for the LRP, we used rabbit β-VLDL, which is a mixture mostly containing VLDL remnants but containing some chylomicron remnants (21). As shown in Fig. 4, when FH 808 cells were incubated with rabbit β-VLDL without added apo E, no

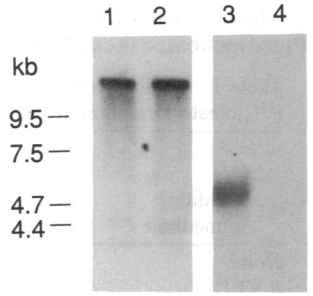

FIG. 2. Blot hybridization of RNA from human fibroblasts. Cells from a normal subject (lanes 1 and 3) and FH 808 (lanes 2 and 4) were cultured without sterols. Total cellular RNA (10 μg) was denatured, subjected to electrophoresis, and transferred to nylon membranes. The membranes were hybridized with ^{32}P-labeled probes (4 × 10^6 cpm/ml) specific for either LRP mRNA (lanes 1 and 2) or LDL receptor mRNA (lanes 3 and 4), washed, and exposed to Kodak XAR-5 film with an intensifying screen for 12 hr at −70°C.

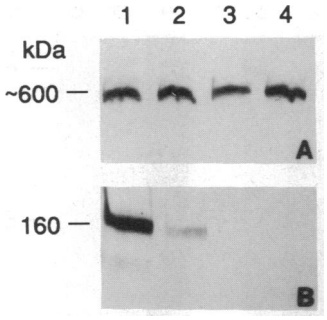

FIG. 3. Biosynthesis of LRP and LDL receptor in human fibroblasts. Cells from a normal subject (lanes 1 and 2) and FH 808 (lanes 3 and 4) were incubated for 18 hr in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 25-hydroxycholesterol at 2 μg/ml plus cholesterol at 10 μg/ml, after which they were metabolically labeled with [^{35}S]cysteine (100 μCi/ml; 1 Ci = 37 GBq) in cysteine-free medium (19). Immunoprecipitation of detergent-solubilized cell extracts was done with either polyclonal anti-LRP IgG and Protein A–Sepharose (6) (*A*) or monoclonal anti-LDL receptor IgG-C7 and immune complexes (20) (*B*). Samples were boiled in the presence of 1% SDS and 100 mM 2-mercaptoethanol and separated on 5% SDS/PAGE. Gels were treated with Entensify (DuPont), exposed to Kodak XAR-5 film for either 7 hr (*A*) or 36 hr (*B*) at −70°C, and calibrated as described for Fig. 1.

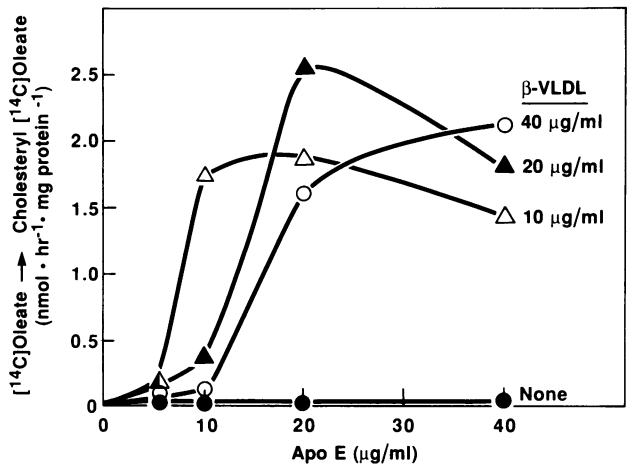

FIG. 4. Stimulation of cholesteryl [^{14}C]oleate formation in FH 808 fibroblasts by β-VLDL and apo E. On day 7 each monolayer received 2 ml of medium A containing the indicated concentration of β-VLDL and apo E that had been previously incubated as described. After 5-hr incubation at 37°C, the cells were pulse-labeled for 2 hr with [^{14}C]oleate-albumin, and their content of cholesteryl [^{14}C]oleate was measured; each value is a single incubation. The amount of cholesteryl [^{14}C]oleate formed in the absence of β-VLDL and apo E was 0.025 nmol·hr^{-1}·mg of protein^{-1}.

detectable stimulation of cholesteryl [^{14}C]oleate synthesis occurred. When β-VLDL was preincubated with increased amounts of apo E, there was progressive increase in the ability of β-VLDL to stimulate cholesteryl [^{14}C]oleate formation. The maximum response was relatively independent of the concentration of β-VLDL, but there was a clear relation between the amount of β-VLDL and the amount of apo E required. Thus, when β-VLDL was at 10 μg of protein per ml, a maximal effect was seen with 10 μg of apo E per ml. On the other hand, with β-VLDL at 40 μg of protein per ml, apo E at 40 mg/ml was required for maximal stimulation. Addition of apo E by itself, without β-VLDL, did not stimulate any cholesteryl [^{14}C]oleate formation.

Table 1 shows that the ability of apo E-enriched β-VLDL to stimulate cholesteryl ester synthesis was abolished when the cells were treated with chloroquine, which inhibits the lysosomal hydrolysis of cholesteryl esters in lipoproteins internalized by receptor-mediated endocytosis (22). In contrast, chloroquine did not affect the ability of 25-hydroxycholesterol to stimulate cholesteryl [^{14}C]oleate formation, confirming that chloroquine was acting by inhibiting the lysosomal hydrolysis of the lipoprotein-bound cholesteryl esters. The apo E-enriched β-VLDL did not significantly affect incorporation of [^{14}C]oleate into [^{14}C]triglycerides (Table 1).

To show that cholesteryl ester uptake in FH 808 cells was mediated by LRP, we incubated the cells with apo E-enriched β-VLDL in the presence of the polyclonal antibody against LRP. Fig. 5*A* shows that treatment with anti-LRP antibody markedly inhibited stimulation of cholesteryl [^{14}C]oleate synthesis; nonimmune IgG had no such effect. Moreover, a polyclonal anti-LDL receptor antibody that blocks stimulation through the LDL receptor did not block the action of the apo E-enriched β-VLDL.

Normal fibroblasts that were induced for maximal LDL receptor expression showed a 6.5-fold greater stimulation of cholesterol [^{14}C]oleate formation than did the FH 808 cells when incubated with apo E-enriched β-VLDL (Fig. 5*B*). This stimulation was inhibited by the anti-LDL receptor antibody, but the anti-LRP antibody had little effect. In other experiments not shown, we found that the stimulation of cholesterol esterification by β-VLDL in normal fibroblasts was not increased by apo E enrichment. Together, these data indicate that the bulk of β-VLDL uptake in normal fibroblasts is mediated by the LDL receptor, which makes contribution of LRP difficult to detect.

DISCUSSION

The current experiments provide evidence that LRP is present on the surface of cultured human fibroblasts and that it mediates the uptake and lysosomal hydrolysis of cholesteryl esters contained in apo E-enriched lipoproteins, with a re-

Table 1. Inhibition by chloroquine of β-VLDL and apo E-mediated stimulation of cholesteryl [^{14}C]oleate formation in FH 808 fibroblasts

Addition to medium	[^{14}C]Oleate incorporation, nmol·hr^{-1}·(mg of protein)$^{-1}$			
	Cholesteryl [^{14}C]oleate		[^{14}C]Triglycerides	
	− Chloroquine	+ Chloroquine	− Chloroquine	+ Chloroquine
None	0.032	0.010	20.7	16.8
β-VLDL	0.015	0.019	18.6	19.0
Apo E	0.025	0.017	17.5	13.3
β-VLDL + apo E	1.12	0.021	14.7	16.6
Sterols	1.28	1.27	17.5	14.3

On day 7 each monolayer received 2 ml of medium A containing the indicated addition (β-VLDL at 20 μg of protein per ml, apo E at 20 μg/ml, or sterols consisting of a mixture of 25-hydroxycholesterol at 5 μg/ml plus cholesterol at 10 μg/ml added in 10 μl of ethanol) with or without 75 μM chloroquine. After 5-hr incubation at 37°C, the cells were pulse-labeled with [^{14}C]oleate-albumin for 2 hr, after which their content of [^{14}C]lipids was measured. Each value is the average of triplicate incubations.

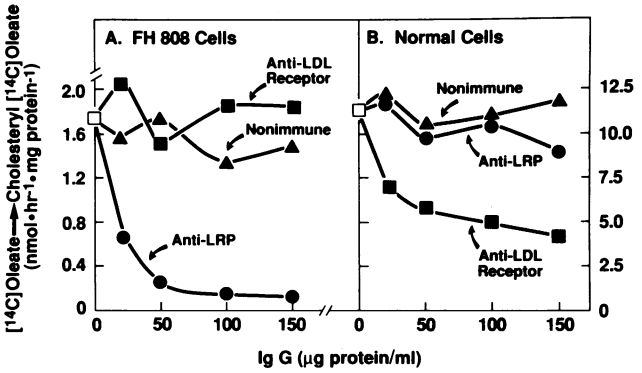

FIG. 5. Inhibition of β-VLDL and apo E-mediated stimulation of cholesteryl [^{14}C]oleate formation in fibroblasts from FH 808 (*A*) and a normal subject (*B*). On day 7 each monolayer received 1.7 ml of medium A containing the indicated concentration of the indicated IgG fraction. After 90-min incubation at 37°C, each dish received 0.3 ml of medium A containing β-VLDL at a final concentration of 25 μg of protein per ml and apo E that had been previously incubated as described at a final concentration of 25 μg/ml. The cells were incubated for 5 hr at 37°C, after which they were pulse-labeled with [^{14}C]oleate-albumin and harvested for measurement of cholesteryl [^{14}C]oleate content. Each value is a single incubation except for those receiving no IgG (□), which are the means of triplicate incubations. Incubation of FH 808 cells in *A* with preimmune IgG at 20–150 μg/ml (prepared from serum of the same rabbit immunized with LRP) showed no inhibition of cholesteryl [^{14}C]oleate formation; the preimmune IgG produced a slight stimulation (data not shown).

sultant stimulation in cellular cholesteryl ester synthesis. The uptake can be blocked by pretreatment of the cells with a polyclonal antibody prepared against the affinity-purified LRP from rat liver. These results, together with previous observations on LRP (6), raise the possibility that LRP may serve as an endocytosis-mediating lipoprotein receptor with activity for large VLDL particles and chylomicron remnants.

An unexpected result of the current studies was the requirement for artificial enrichment of β-VLDL with excess apo E in order to achieve functional LRP binding. The β-VLDL used in these studies contains a relatively large amount of apo E, and the β-VLDL binds with high affinity to LDL receptors (Fig. 5*B*) and to macrophage β-VLDL receptors (data not shown). Nevertheless, the β-VLDL does not seem to bind to LRP in amounts sufficient to stimulate cholesteryl ester synthesis in FH 808 cells (Fig. 4). Windler and Havel (23) showed that binding of apo E to LDL receptors and to hepatic chylomicron remnant receptors is inhibited by the C apoproteins. Perhaps a greater excess of apo E in relation to apo C is required for ligand binding to the LRP than to the LDL receptor. We cannot rule out the possibility that β-VLDL *in vivo* contains sufficient apo E for binding to LRP and that this is removed during the isolation procedure. However, this explanation seems unlikely because β-VLDL accumulates in the plasma of cholesterol-fed rabbits. If this particle were fully active in binding to LRP, it should be cleared from the circulation of the cholesterol-fed rabbit.

Inasmuch as β-VLDL is heterogeneous in size and composition, it is possible that only a subpopulation of β-VLDL can bind to the LRP when enriched with apo E. On a molar basis, ≈15% of the apo B in β-VLDL was in the form of B-48 and 85% was in the form of B-100, as judged by densitometry of Coomassie-stained SDS gels (data not shown). We do not know whether both populations of particles bind to LRP. The stoichiometry of binding in the FH 808 cells can be estimated by comparison with LDL binding in normal cells. The ratio of total cholesterol to protein in the β-VLDL preparation used in Figs. 4 and 5 is 10:1. To achieve the observed stimulation of cholesterol esterification, the FH 808 cells must have been taking up ≈800 ng of cholesterol per hr per mg of cell protein, which equals 80 ng of protein per hr per mg of cell protein. This is <0.1 the rate of LDL uptake at saturation in normal fibroblasts (12). The uptake and degradation of this amount of protein is too small to be reliably detected by our current assays for ^{125}I-labeled β-VLDL. We believe that this insensitivity of the assay accounts for our inability to measure the direct binding, uptake, or degradation of ^{125}I-labeled apo E-enriched β-VLDL in the FH 808 cells. In normal fibroblasts induced for LDL receptor expression, β-VLDL caused a maximal stimulation of cholesteryl [^{14}C]oleate formation that was ≈6-fold higher than the amount seen with the apo E-enriched β-VLDL in the FH 808 cells (compare Fig. 5 *A* and *B*). In normal cells we could not detect any additional stimulation by added apo E, indicating that the vast bulk of β-VLDL uptake in normal fibroblasts is mediated by the LDL receptor, which does not require added apo E. Note that our apo E-enriched β-VLDL is an arbitrarily produced ligand. LRP may bind its natural ligand with higher affinity and deliver it to lysosomes with greater efficiency.

Because of our inability to measure the binding, uptake, and degradation of apo E-enriched ^{125}I-labeled β-VLDL to cells, we cannot be certain that the effect of apo E is exerted on the binding reaction itself. It is possible that the apo E affects the intracellular routing of the internalized β-VLDL in such a way that its cholesteryl esters are delivered more efficiently to lysosomes. We also do not know whether the anti-LRP antibody inhibits cholesteryl ester uptake by blocking the binding of the apo E-enriched β-VLDL or by causing the receptor to become sequestered within the cell. It is likely that all of these quesitons may be answered by studies of cells that produce high levels of LRP as a result of transfection with a cDNA expression vector.

Eisenberg *et al.* (24) recently showed that enrichment of normal human VLDL with apo E *in vitro* markedly increased its ability to bind to LDL receptors of normal human fibroblasts, but they found no detectable binding to FH homozygote cells. It is possible that apo E-enriched VLDL isolated from normolipidemic human subjects does not bind to LRP or, alternatively, that the assays using ^{125}I-labeled lipoproteins were not sensitive enough to detect LRP activity in the FH cells (see above discussion). Oswald and Quarfordt (25) showed that addition of apo E to a triglyceride emulsion increased its uptake by isolated hepatocytes and HepG2 cells, an effect that was blocked by apo C proteins. It seems likely that this uptake may have been mediated both by the LDL receptor and LRP.

The current studies raise the interesting possibility that LRP may not bind lipoproteins in the form in which they normally circulate in plasma. Binding to LRP may require preliminary sequestration of the lipoproteins in a tissue space within which they can absorb excess apo E. Such a space is found in tissues with a fenestrated capillary endothelium, such as the space of Disse in the liver and the subendothelial space of the adrenal gland. Both of these organs are very active in the production of apo E (26) as well as in the uptake of chylomicron remnants (2), and the LRP may be involved in such uptake.

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