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# Inefficient Degradation of Triglyceride-rich Lipoprotein by HepG2 Cells Is Due to a Retarded Transport to the Lysosomal Compartment*

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**Binding studies at 37 °C showed that lipoprotein lipase-treated very low density lipoproteins (LPL-VLDL) and very low density lipoproteins (VLDL), once taken up via the low density lipoprotein (LDL) receptor, are poorly degraded by HepG2 cells as compared with LDL. Determination of the initial endocytotic rate for LPL-VLDL and VLDL as compared to LDL shows that LPL-VLDL and VLDL are internalized at a similar rate as LDL. Incubation of cells with labeled LDL, LPL-VLDL, and VLDL at 18 °C for 4.5 h resulted in the accumulation of these particles in the early endosomes, without subsequent transport to the lysosomes and degradation. After washing the cells and a temperature shift to 37 °C, the labeled LDL present in the early endosomes is transported to the lysosomal compartment almost completely within 15 min. Strikingly, for LPL-VLDL and for VLDL, only about 50% or less of the label was moved to the lysosomal compartment within 45 min. However, once present in the lysosomes, VLDL and LPL-VLDL are degraded about 1.6-fold more rapidly than LDL.**

**Retroendocytosis accounts for less than 10% of the internalized LDL, whereas a higher rate of retroendocytosis, up to 20 and 40%, respectively, was observed for LPL-VLDL and VLDL.**

**To evaluate the effect of the inefficient transport of VLDL and LPL-VLDL to the lysosomal compartment on cellular cholesterol homeostasis, acyl-CoA:cholesterol acyltransferase (ACAT) activity was measured. Incubation with 30 μg/ml of LDL induced a 2.5-fold increase in ACAT activity, whereas the incubation with similar amounts of both VLDL and LPL-VLDL failed to stimulate this enzyme.**

**We conclude that both a slower transport to the lysosomal compartment and a higher rate of retroendocytosis, possibly as the consequence of the longer residence time in the early endosomes, are responsible for the poor degradation of VLDL and LPL-VLDL by HepG2 cells.**

Very low density lipoprotein (VLDL)[1] are triglyceride-rich, apolipoprotein (apo) E- and apoB100-containing, lipoprotein particles that are synthesized and secreted by the liver. After entering the bloodstream, VLDL particles interact with lipoprotein lipase (LPL), which catalyzes the hydrolysis of triglycerides. The resulting remnant particles are smaller, more dense, and have an altered lipid and apolipoprotein composition, as compared with native VLDL particles (for review, see Ref. 1). The VLDL remnants are further lipolysed and converted into intermediate density lipoproteins and, finally, low density lipoproteins (LDL). During VLDL lipolysis, a fraction of the remnants is directly cleared from the plasma via hepatic LDL receptors, where apoE, the major protein constituent of these particles, acts as a ligand (2–6).

Many lipoprotein particles that contain apoE have several copies of this protein and are thought to react more avidly with the LDL receptor than LDL (7, 8). A single lipoprotein particle containing several molecules of apoE could interact multivalently with a single LDL receptor; alternatively, lipoproteins containing several molecules of apoE may interact with more than one LDL receptor. In either case, particles that contain apoE in addition to apoB100 will bind to the LDL receptor with higher affinity than those that contain only one apoB100 molecule (9).

Recent studies by Tabas *et al.* (10) have shown that the multivalent binding of β-VLDL through apoE to the LDL receptor in mouse peritoneal macrophages leads to a divergent endocytotic pathway as compared to LDL. They found that LDL is rapidly targeted to perinuclear lysosomes near the center of the cell, whereas, after its uptake, β-VLDL is localized in more distributed vesicles. This differential distribution was found to be coupled to a slower degradation of β-VLDL concomitant with a higher capability to stimulate acyl-CoA:cholesterol acyltransferase (ACAT).

In previous studies we found that the degradation of VLDL and LPL-treated VLDL, representing VLDL-remnants, by HepG2 cells is extremely low as compared to that of LDL.[2] A low degradation efficiency of VLDL has also been described by other investigators (11). In the present study, we addressed the question as to whether this inefficient degradation might be due to an altered intracellular processing of these particles, possibly due to their multivalent binding via apoE. The present results clearly show that after internalization, the transport of VLDL as well as of LPL-treated VLDL to the lysosomal compartment is indeed severely retarded, whereas, once present in the lysosomes, these particles are catabolyzed about 1.6-fold more rapidly than LDL. In addition, we found that these lipoproteins fail to stimulate intracellular ACAT activity.

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[1] The abbreviations used are: VLDL, very low density lipoproteins; ACAT, acyl-CoA:cholesterol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HSA, human serum albumin; LDL, low density lipoproteins; LPDS, lipoprotein-depleted serum; LPL, lipoprotein lipaseL-VLDL, lipoprotein lipase-treated VLDL; PBS, phosphate-buffered saline; HTG, hypertriglyceridemic.

[2] P. Lombardi, M. Mulder, H. Van der Boom, R. R. Frants, and L. M. Havekes, unpublished observations.

## EXPERIMENTAL PROCEDURES

*Materials*—Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM, cell culture medium) were obtained from Flow Laboratories (Irvine, United Kingdom). Human serum albumin (HSA) was obtained from Sigma. Na$^{125}$I (specific activity 13.3 μCi/μl) was purchased from Amersham (Buckinghamshire, U.K.). Multiwell cell culture dishes were from Costar (Cambridge, MA). Proteinase K was purchased from Boehringer Manheim (Manheim, Germany). Percoll (density 1.13 g/ml) was obtained from Pharmacia (Uppsala, Sweden).

*Lipoproteins*—LDL and VLDL were isolated from serum of normolipidemic donors by density gradient ultracentrifugation according to Redgrave *et al.* (12). Lipoprotein lipase-treated VLDL (LPL-VLDL) were prepared by incubating total serum with LPL purified from bovine milk (13), essentially as described before (5). Briefly, the amount of LPL added was equal to the amount necessary for hydrolysis of 50% of the triacylglycerols present in complete serum within 1 h. The incubation was performed in the presence of 10% (w/v) fatty acid-free HSA and Tris-HCl buffer (final concentration 0.1 M, pH 8.5) for 90 min at 37 °C. To stop the reaction, the mixture was put on ice and solid KBr was added to adjust the solution to a density of 1.21 g/ml. LPL-VLDL, with density less than 1.019 g/ml, were then isolated by density gradient ultracentrifugation (12).

The lipoprotein preparations were immediately used for iodination by the $^{125}$iodine monochloride method described by Bilheimer *et al.* (14). After iodination, the lipoproteins were dialyzed against phosphate-buffered saline (PBS, pH 7.4) and stabilized with 1% (w/v) HSA. The specific activities ranged from 100 to 250 counts/min/ng of protein. The stabilized $^{125}$I-labeled lipoproteins were stored at 4 °C and used within 2 weeks. With all labeled lipoproteins, less than 1% of the radioactivity was soluble in 10% (w/v) trichloroacetic acid. Whenever unlabeled lipoproteins were used, they were immediately stabilized after isolation with 1% (w/v) HSA followed by extensive dialysis against PBS and, subsequently, DMEM supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml).

Lipoprotein-depleted serum (LPDS) was obtained by ultracentrifugation of serum at a density of 1.21 g/ml followed by extensive dialysis of the infranatant against PBS and, subsequently, DMEM supplemented with penicillin and streptomycin.

β-VLDL was separated by sequential ultracentrifugation from the serum of cholesterol-fed rabbits (d < 1.006 g/ml) and extensively dialyzed against PBS and, subsequently, DMEM supplemented with penicillin and streptomycin.

*Cell Culture*—HepG2 cells were cultured in 25-cm$^2$ flasks in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 20 mM HEPES, 10 mM $NaHCO_3$, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in equilibration with 95% air, 5% $CO_2$. Six to 7 days prior to each experiment, cells were seeded in 2-cm$^2$ multiwell dishes.

Twenty-four h before the assays, the cells were washed with DMEM-1% HSA and further incubated with DMEM containing 10% LPDS (v/v) instead of FCS.

*Receptor-mediated Cell Association and Degradation*—Receptor-mediated cell association and degradation were measured essentially as described previously (5, 15).

Each experiment was started by washing the cells three times in DMEM, 1% HSA. Thereafter, cells were incubated in the same medium with the addition of 20 μg/ml of labeled lipoproteins, in the presence or absence of a 30-fold excess of unlabeled LDL. Temperature and duration of the respective incubations are described in the text and figure legends. After incubation with labeled lipoproteins, cells were cooled to 0 °C. Degradation was measured exactly as described (16) and, after removal of the incubation medium, the cells were washed extensively (17). To measure total cell association, the washed cells were dissolved in 0.2 M NaOH, and an aliquot of the cell lysate was counted for radioactivity. Another aliquot of the cell lysate was used for protein determination according to Lowry *et al.* (18).

Values for the receptor-mediated cell association and degradation were calculated by subtracting the amount of labeled lipoprotein that was cell associated or degraded in the presence of a 30-fold excess of unlabeled LDL (nonspecific binding) from the amount of labeled lipoprotein that was cell-associated or degraded in the absence of an excess of unlabeled LDL (total binding).

*Initial Rate of Endocytosis*—To measure the initial rate of endocytosis, cells were incubated for the indicated periods of time at 37 °C in DMEM, 1% HSA with the addition of 20 μg/ml of labeled lipoproteins, in the presence or absence of a 30-fold excess of unlabeled LDL. Cells were then washed extensively and further incubated with 0.5 mg/ml proteinase K, 1 mM EDTA in PBS for 1 h at 0 °C. The protease activity was then neutralized by adding the same volume of PBS containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The detached cells were collected by centrifugation at 150 × *g* for 5 min at 0 °C. The radioactivity released into the buffer represents the amount of $^{125}$I-labeled lipoprotein bound to the cell membrane. The radioactivity that remains cell associated represents the amount of $^{125}$I-labeled lipoprotein internalized.

Values for the receptor-mediated binding and internalization were calculated by subtracting the amount of labeled lipoprotein that was bound or internalized in the presence of a 30-fold excess of unlabeled LDL from the amount of labeled lipoprotein that was bound or internalized in the absence of an excess of unlabeled LDL.

*Measurement of Intracellular Transport of Lipoproteins*—Subcellular fractionation of HepG2 cells was performed by Percoll density gradient centrifugation as described (19). Cells, seeded in 60- or 100-mm dishes, were incubated in the presence of 20 μg/ml of $^{125}$I-labeled LDL, LPL-VLDL, or VLDL. Temperature and duration of the respective incubations are described in the text and figure legends. After incubation with the labeled lipoproteins, cells were cooled to 0 °C. The incubation medium was removed and degradation measured as described (16). Cells were then washed extensively with 0.28 M sucrose, 2 mM $CaCl_2$, 0.01 M Tris-HCl, pH 7.6 and scraped from the dishes with a rubber policeman in the same buffer (1 ml/dish). Then, cells were homogenized in a Dounce homogenizer by 20 complete strokes with a tight fitting pestle. The homogenates were centrifuged at 280 × *g* for 10 min in order to remove remaining intact cells. A 80% (v/v) Percoll solution and homogenization buffer were added to the supernatants to a final Percoll concentration of 20% and to a final volume of 12 ml. After thorough mixing, the samples in Percoll were placed in cellulose nitrate tubes fitting a Ti-50 rotor (Beckman) and centrifuged at 10,000 × *g* for 45 min. Fractions of ~0.3 or 0.5 ml were collected by aspiration from top to bottom, and the radioactivity in each sample was counted. The density of each fraction was measured in a PAAR-DMA-45 density meter equipped with a DMA-602M small sample cell (~170 μl). The distribution of the lysosomal marker (acid phosphatase) was measured by the method of Torriani (20).

*Measurement of Intracellular Cholesterol Esterification (ACAT Activity)*—Cholesterol esterification was measured by determining the incorporation of [1-$^{14}$C]oleic acid into labeled cholesteryl oleate, essentially as described (21), with some minor modifications. Cells cultured in 10-cm$^2$ wells were first preincubated for 20 h in culture medium containing 10% LPDS and then with the indicated amounts of lipoproteins for 4 h. Subsequently, to 2 ml of incubation medium, 100 μl of a 1 mM solution of [$^{14}$C]oleate (2340 disintegrations/min/nmol) complexed to albumin was added, and cells were incubated for another 2 h at 37 °C. Cells were then washed four times with ice-cold PBS and harvested in 1 ml by scraping. After addition of [$^3$H] cholesterol (60,000 disintegrations/min/sample), as internal standard, lipid extraction as described by Bligh and Dyer (22) was performed. Lipids were analyzed by thin layer chromatography on precoated silica plates. The developing solvent was composed of chloroform/methanol (98:2) (v/v) first, followed by chloroform/hexane (45:65) (v/v). The lipid spots were detected by autoradiography, scraped off, and counted for radioactivity in a Packard 1900CA Tri-Carb liquid scintillation analyzer equipped with software validated for $^{14}$C/$^3$H double-labeled samples. Recovery of the internal standard was 65–85%.

## RESULTS

*Time Course of Receptor-mediated Association and Degradation of LDL, LPL-treated VLDL, and VLDL by HepG2 Cells*—The time course of receptor-mediated association and degradation of labeled LDL, LPL-VLDL, and VLDL at 37 °C are shown in Fig. 1. For all three lipoprotein samples, the cell-association increased progressively over the first 3 h, before a plateau is reached. The degradation started after a lag period of 60–90 min and proceeded at a slower rate in case of both LPL-VLDL and VLDL, as compared with LDL. When the degradation efficiency is calculated as the amount of lipoprotein degraded relative to the amount of lipoprotein that became cell-associated (Fig. 2), it is apparent that after 5 h of incubation the degradation efficiency of LPL-VLDL and VLDL is only 50 and 20%, respectively, of that of LDL.

We reasoned that more information about the intracellular

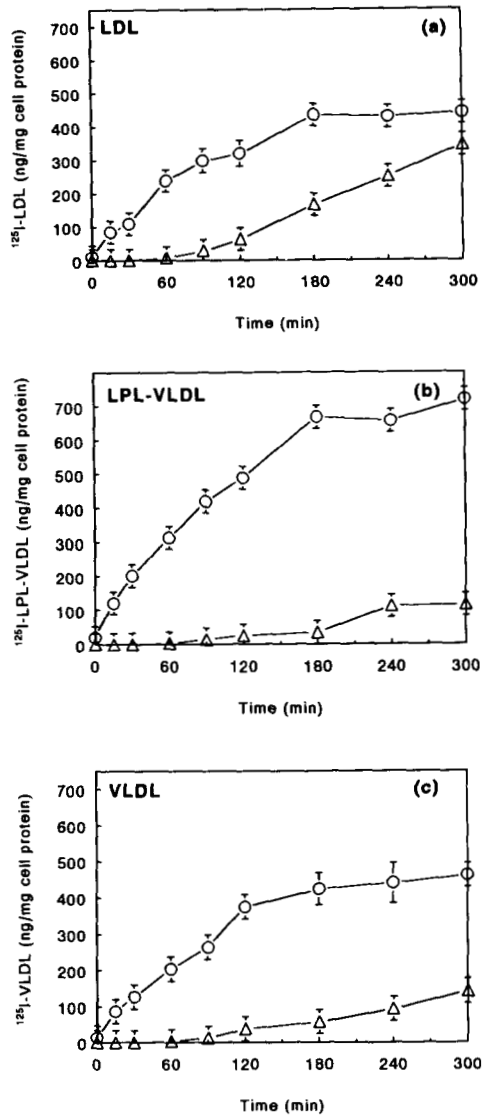


FIG. 1. **Time course at 37 °C of the receptor-mediated association (○) and degradation (△) of LDL (*a*), LPL-VLDL (*b*), and VLDL (*c*) in HepG2 cells.** Cells were incubated with 20 μg/ml of $^{125}$I-LDL or $^{125}$I-LPL-VLDL or $^{125}$I-VLDL ± 30-fold excess of unlabeled LDL at 37 °C for the indicated periods of time. Thereafter, the receptor-mediated association and degradation were measured as described under "Experimental Procedures." Values are means ± S.D. of triplicate incubations.

processing of LPL-VLDL and VLDL might help explain this difference in degradation efficiency. Therefore, the next experiments were designed to investigate whether the reduced degradation efficiency of LPL-VLDL and VLDL was due to (i) a lower rate of endocytosis, (ii) a less efficient transport of the apoE-binding lipoproteins from the early endosomal compartment to the lysosomal compartment, or (iii) an impairment in the lysosomal degradation itself.

*Initial Rate of Endocytosis of Surface-bound LDL, LPL-VLDL, and VLDL by HepG2 Cells*—The initial endocytotic rate of LDL, LPL-VLDL, and VLDL was studied as described by Wiley and Cunningham (23) (Fig. 3). Cells were incubated with 20 μg/ml of labeled lipoproteins. At the specified time points, cells were extensively washed, and the ratio of radioactivity associated with the interior of cells (internalized) to that associated with the surface (bound) was measured. Fig. 3 shows that the rate of endocytosis of LPL-VLDL and VLDL is similar to that of LDL. We hypothesize therefore that LPL-

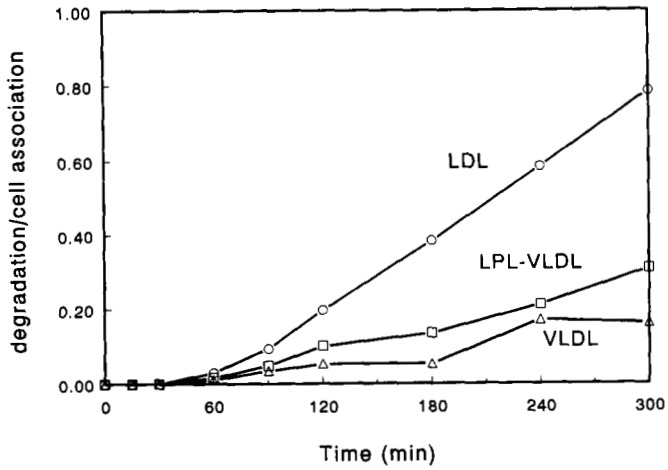


FIG. 2. **Degradation efficiency of LDL (○), LPL-VLDL (□), and VLDL (△).** Values are calculated from the results presented in Fig. 1 as ratio degradation/association

VLDL and VLDL, once internalized at a normal rate (i) cannot be further transported to the lysosomal compartment, or (ii) they cannot be degraded in the lysosomes either due to an impairment in the late endosome-lysosome fusion or to a defect in the lysosomal degradation itself.

*Intracellular Processing and Rate of Retroendocytosis of LDL, LPL-VLDL, and VLDL*—To evaluate whether the transport of LPL-VLDL and VLDL from the early endosomal compartment to the late endosomal or lysosomal compartment is impaired, cells were first incubated with labeled lipoproteins for 4.5 h at 18 °C. At this temperature, it has been demonstrated that degradation of LDL is inhibited owing to an impairment in the dissociation of the internalized LDL from the receptor (24) and to a block in endosome-lysosome fusion (25). As a result, the cell-associated lipoproteins will accumulate in the early endosomal compartment, without being degraded (26).

After the incubation in the presence of labeled LDL, LPL-VLDL, or VLDL at 18 °C, cells were washed in order to remove the unbound ligand and further incubated at 37 °C for the indicated periods of time (Fig. 4). With LPL-VLDL and VLDL the major portion of the initial amount of label accumulated in the endosomes is still cell associated after 5 h at 37 °C (Fig. 4, *b* and *c*), whereas for LDL (Fig. 4*a*) about 70% of the internalized LDL is degraded within 5 h after the temperature shift from 18 to 37 °C. For LDL, the decrease in cell association is fully complementary to the amount of LDL degraded. This implies that all intracellularly present LDL is released, after degradation. Strikingly, for LPL-VLDL and VLDL the sharp decline of the cell association curve in the first hour of incubation after the temperature shift from 18 to 37 °C suggests that part of the intracellularly accumulated particles are excreted as intact particles into the medium (retroendocytosis), thus escaping the degradation route, a process which has been found to account for up to 10% of the internalized LDL (27). To further investigate this possibility, retroendocytosis was calculated from the data reported in Fig. 4. Indeed, we found that retroendocytosis (Fig. 4, *broken line*) accounts for less than 10% of the initially internalized LDL, whereas up to 20 and 40% of LPL-VLDL and VLDL, respectively, appear to be released intact into the medium.

These data indicate that the inefficient degradation of LPL-VLDL and VLDL is the result of the combined effect of an impaired process downstream the early endosomal compartment, and of retroendocytosis that diverts a substantial

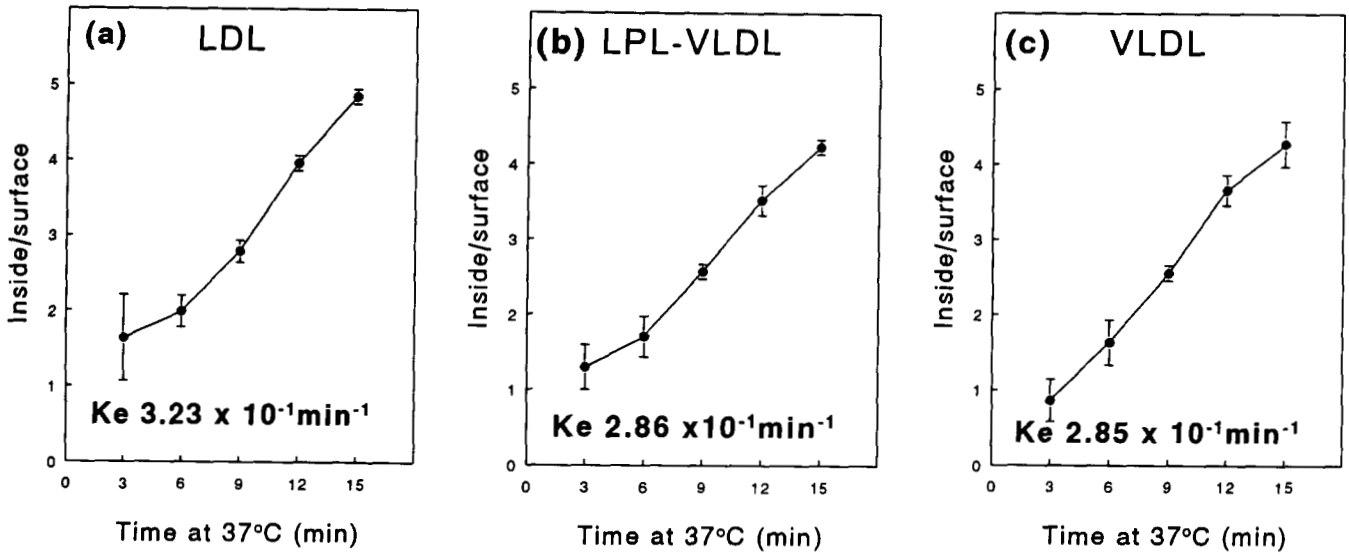


FIG. 3. **Initial endocytotic rate of LDL (*a*), LPL-VLDL (*b*), and VLDL (*c*) in HepG2 cells.** Cells were incubated at 37 °C with 20 μg/ml of $^{125}$I-LDL, $^{125}$I-LPL-VLDL, or $^{125}$I-VLDL ± 30-fold excess of unlabeled LDL. At the indicated time ponts, cells were quickly washed at 0 °C, and the ratio of radioactivity internalized to that bound to the surface was determined as outlined under "Experimental Procedures." Values are means ± S.D. of triplicate incubations. *Ke* indicates the endocytotic rate constant.

amount of the internalized LPL-VLDL and VLDL from the intracellular processing.

*Rate of Transport of LDL, LPL-VLDL, and VLDL from the Early Endosomes to the Lysosomes*—To investigate as to whether the intracellularly accumulated LPL-VLDL and VLDL are either retained in the sorting endosomes or normally delivered to the lysosomal compartment, but not further degraded, cells were incubated for 4.5 h at 18 °C in the presence of labeled lipoprotein, followed by a temperature shift to 37 °C and homogenization at the indicated time points. Thereafter, cell homogenates were subcellularly fractionated (Fig. 5) (19). Due to their difference in buoyant density, the early and sorting endosomes (*top fractions*) were separated from the lysosomal fractions (*bottom fractions*) by Percoll-gradient centrifugation. The late endosomes, having a density similar to that of the lysosomes (28), are recovered with the high density fraction. Since a further discrimination between the late endosomal and the lysosomal compartment is not feasible with the method selected for subcellular fractionation, we will refer to the high density fractions as those representing the late endosomal-lysosomal compartment. Fig. 5 shows the distribution of label in the gradient fractions for each lipoprotein tested at one time point (15 min) after the temperature shift. With LDL (Fig. 5*a*), after 15 min at 37 °C, almost all the radioactivity was found in the high density bottom fractions, which represent the late endosomal fractions and lysosomal fractions. The latter were identified by the presence of acid phosphatase activity (*horizontal bar*). Strikingly, LPL-VLDL, and even more dramatically VLDL, move much more slowly to the bottom fractions upon incubation at 37 °C. After 15 min at 37 °C, more than 50% of LPL-VLDL (Fig. 5*b*) and almost all VLDL (Fig. 5*c*) was still present in the light, early endosomal fractions. The rate of accumulation of the labeled lipoproteins in the high density fractions at different time points is summarized in Fig. 6. Within 15 min after the temperature shift to 37 °C, the intracellular trafficking of LDL toward the late endosomal-lysosomal compartment was nearly complete, while for LPL-VLDL and VLDL, even after 45 min, the entire process toward the late endosomal-lysosomal compartment has not been completed. When the presence of labeled particles in the high density fractions was measured at longer time intervals (up to 90 min), the rate of accumulation declined, as a result of the increase in the amount of lipoprotein degraded (not shown). Apparently, LPL-VLDL and VLDL are much more slowly transported to the late endosomes or lysosomes than LDL.

*Rate of Degradation of LDL, LPL-VLDL, and VLDL in the Lysosomes*—Fig. 5 indicates that, although at a slower rate, a substantial amount of LDL-VLDL and VLDL reaches the lysosomes. This would suggest that next to a slower transport to the lysosomes a slower turnover of LPL-VLDL and VLDL in the lysosomes might also contribute to the overall effect of a sluggish catabolism of these particles. To verify this hypothesis, cells were incubated with labeled LDL, LPL-VLDL, and VLDL at 37 °C for 5 h. At this time point cells are assumed to have reached a steady-state, as evaluated from the cell association curve shown in Fig. 1. Cells were then rapidly washed at 37 °C with prewarmed medium and further incubated for 30 min at 37 °C in the presence of the same amount of labeled lipoproteins as in the previous incubation. The medium was collected for measuring degradation, after which cells were cooled to 0 °C, washed extensively, homogenized, and subjected to subcellular fractionation. The rate of degradation was calculated as the ratio of the amount of lipoprotein degraded in 30 min over the amount of lipoprotein present in the lysosomal fractions at steady-state. The results presented in Table I show that, once present in the lysosomes, LPL-VLDL and VLDL are degraded even more efficiently than LDL. Thus an impaired lysosomal degradation itself is not responsible for the observed low degradation efficiency of VLDL and LPL-VLDL by HepG2 cells (Figs. 1 and 2).

*Cellular Cholesterol Esterification (ACAT Activity)*—In order to determine if there was a correlation between the retarded transport of LPL-VLDL and VLDL to the late endosomal or lysosomal compartment and the potency of these particles to stimulate ACAT, ACAT activity was measured after incubation of HepG2 cells with either LDL, LPL-VLDL, VLDL, or rabbit β-VLDL. In macrophages, β-VLDL are known to be a much more potent stimulator of ACAT than LDL, although this effect is not due to a greater delivery to the cell of β-VLDL cholesterol (29). As shown in Fig. 7, after

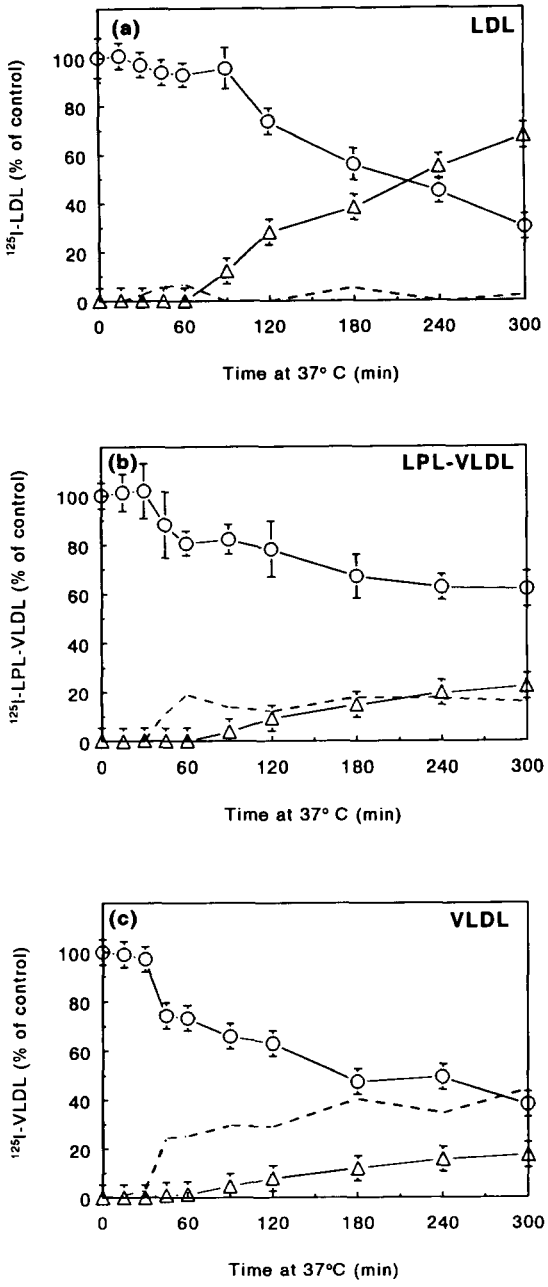


FIG. 4. **Intracellular processing and rate of retroendocytosis of LDL (*a*), LPL-VLDL (*b*), and VLDL (*c*).** Cells were preincubated at 18 °C for 4.5 h in the presence of 20 μg/ml of $^{125}$I-labeled lipoproteins ± 30-fold excess of unlabeled LDL and then chased for the indicated periods of time at 37 °C. Receptor-mediated association (○) and degradation (△) were measured. The amount of lipoprotein associated at time 0 was taken as 100% (control value). The 100% values of the cell-association at time 0 at 37 °C are 74 ± 8, 127 ± 4, 62 ± 4 ng/mg cell protein for LDL, LPL-VLDL, and VLDL, respectively. At each time point the retroendocytosis rate for LDL, LPL-VLDL, and VLDL, (- - -) was calculated according to the formula: retroendocytosis = 100% − (% lipoprotein cell associated + % lipoprotein degraded). Values are means ± S.D. of triplicate incubations.

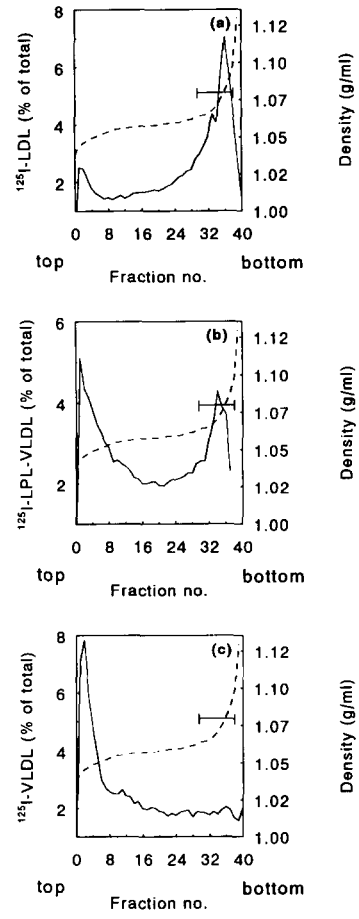


FIG. 5. **Subcellular distribution of LDL (*a*), LPL-VLDL (*b*), and VLDL (*c*) in HepG2 cells.** Cells were incubated for 4.5 h at 18 °C with 20 μg/ml $^{125}$I-labeled lipoprotein in DMEM, 1% HSA medium. After washing to remove unbound ligand, the cells were incubated at 37 °C for 15 min after which the cells were homogenized and subjected to subcellular fractionation as described under "Experimental Procedures." Fractions were measured for radioactivity. The *dotted line* represents the density profile of the gradient; the *horizontal bar* indicates the samples representing the lysosomal fractions as evaluated by acid phosphatase activity measurement.

6 h of incubation with 30 μg/ml of LDL, a 2.5-fold increase of the enzyme activity was obtained, as compared to the control level of ACAT activity in HepG2 cells. Similar amounts of β-VLDL stimulated ACAT up to 5-fold. LPL-VLDL and VLDL did not influence cellular ACAT activity at all. The same results were obtained when cells were incubated for a prolonged time (20 h instead of 6 h) and in the presence of higher amounts of lipoprotein (up to 150 μg of lipoprotein protein/ml). Results similar to those presented in Fig. 7 were obtained when the amount of lipoprotein added was expressed as microgram cholesterol/milliliter. The poor ability of LPL-VLDL and VLDL to stimulate ACAT is in accordance with the retarded transport of these particles to the late endosomal/lysosomal compartment.

## DISCUSSION

In the present study we have shown that normal VLDL and lipolysed VLDL, representing VLDL remnants, once bound and taken up by the LDL receptor in HepG2 cells, are poorly degraded as compared to LDL. A low degradation efficiency has also previously been reported for both VLDL (11) and VLDL remnants (30). In the latter study, the authors propose that either a rapid dissociation of intermediate density lipoprotein-receptor complexes at the cell surface might take place, prior to internalization, or intermediate density lipoprotein might be internalized but a major fraction recycles back to the cell surface (retroendocytosis), possibly together with the receptor protein, thus preventing the routing to the lysosomes. Our present data rule out the first hypothesis, clearly showing that the rate of endocytosis of VLDL and

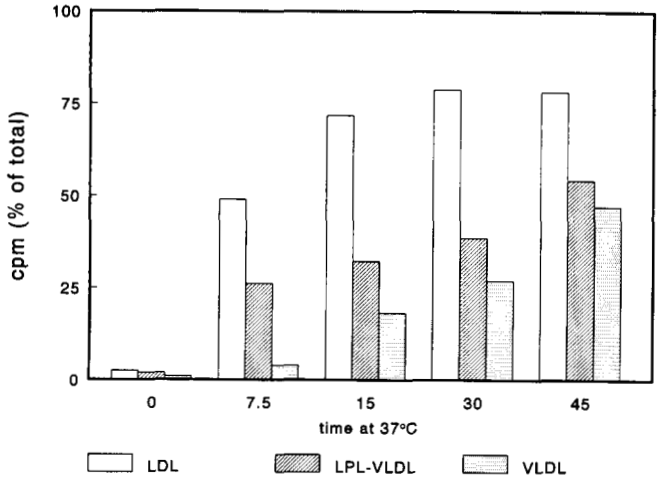


FIG. 6. **Accumulation of LDL, LPL-VLDL, and VLDL in the late endosomal and lysosomal fractions as a function of time.** Cell homogenates were fractionated on Percoll gradients as described in Fig. 5. For each time point the fractions representing the lysosomal fractions (fractions 30–38, see Fig. 5) were pooled and expressed as the percentage of the total amount of radioactivity present in the homogenate. *Open bars* represent LDL; *hatched bars* represent LPL-VLDL, and *dotted bars* represent VLDL.

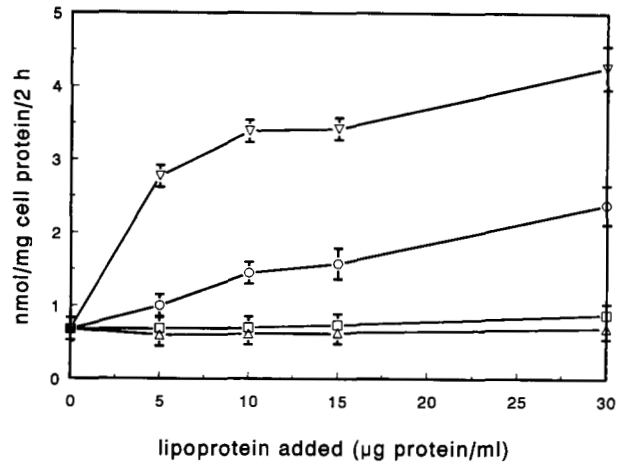


FIG. 7. **Cellular cholesterol esterification (ACAT activity) upon incubation with different lipoprotein fractions.** Cells were incubated for 6 h with DMEM, 10% LPDS containing the indicated amounts of rabbit β-VLDL (▽), LDL (○), LPL-VLDL (□), and VLDL (△). During the last 2 h of the 6-h incubation at 37 °C, to the cells [$^{14}$C]oleate (2340 disintegrations/min/nmol) was added. At the end of the 6-h incubation, the cells were assayed for cholesteryl [$^{14}$C] oleate content as described under "Experimental Procedures." Values are means ± S.D. of triplicate incubations.

TABLE I

*Turnover rate of LDL, LPL-VLDL, and VLDL in lysosomes*

Cells were incubated in the presence of 20 μg/ml of $^{125}$I-labeled LDL, LPL-VLDL, or VLDL at 37 °C for 5 h. Cells were then rapidly washed at 37 °C with prewarmed medium and further incubated for 30 min at 37 °C in the presence of the same amount of labeled lipoproteins as in the previous incubation. Cells were cooled to 0 °C, washed extensively, homogenized and subjected to subcellular fractionation as described under "Experimental Procedures."

| | Lipoprotein degraded[a]/lipoprotein present in the lysosomes[b] |
|---|---|
| LDL | 1.064 ± 0.063[c] |
| LPL-VLDL | 1.61 ± 0.019 |
| VLDL | 1.59 ± 0.024 |

[a] Expressed in ng/mg cell protein/30 min.
[b] Measured as radioactivity present in the high density fractions of Percoll gradients and expressed as ng/mg cell protein.
[c] Values are means ± S.D. of duplicate incubations.

LPL-VLDL is similar to that of LDL (Fig. 3). In case of LPL-VLDL and VLDL, retroendocytosis accounts to a varying extent (from 20 up to 40%) for the amount of label which does not reach the lysosomal compartment. This suggests that a substantial amount of lipoproteins, especially VLDL, is diverted from the routing to the lysosomes and is excreted as intact particles into the medium (Fig. 4). In addition, the fraction of LPL-VLDL and VLDL that does not undergo retroendocytosis is only slowly transported to the lysosomal compartment, whereas, once present in the lysosomes, LPL-VLDL and VLDL are degraded 1.6-fold more rapidly than LDL. This implies that LPL-VLDL and VLDL reside for a longer time than LDL in the early endosomal compartment, thus increasing the probability that they return to the cell surface by retroendocytosis.

Therefore, both a slower transport to the lysosomal compartment and a higher rate of retroendocytosis, possibly as the consequence of the longer residence time in the early endosomes, are responsible for the poor degradation of VLDL and LPL-VLDL by HepG2 cells.

The retarded intracellular routing of these particles might be the result of the polyvalent binding of apoE in VLDL and LPL-VLDL to the receptor. Recently, such a mechanism has been postulated for β-VLDL in mouse peritoneal macrophages (10). It is hypothesized that the high affinity polyvalent apoE binding to the LDL receptor results in a greater resistance to the acid-mediated release of the ligand from the receptor. If this is the case, the rate-limiting step in the processing of VLDL and LPL-VLDL indeed would take place in the sorting endosomes, thus raising the question of the fate of the receptors bound to the ligand. Previous studies have indicated that receptor cross-linking can block ligand-receptor recycling (31, 32), sometimes triggering the delivery of the multivalent-bound receptors to the lysosomes for degradation. Our results, however, cannot discriminate between the two possibilities that either the receptor is relatively slowly recycled back to the plasma membrane or, eventually, partly degraded in the lysosomes.

In order to verify the effect of the slower processing and degradation of VLDL and LPL-VLDL on cellular cholesterol homoeostasis, we measured ACAT activity, which is known to be a sensitive measure for the amount of cholesterol in the regulatory cellular cholesterol pool. Eisenberg *et al.* (11) and Krul *et al.* (33) have found that incubation of cells with VLDL did not lead to a stimulation of ACAT activity. Our results are in line with their results. Both VLDL and LPL-VLDL were not able to stimulate the intracellular cholesterol-esters synthesis (Fig. 7). In contrast with this, Krul *et al.* (33) and Evans *et al.* (34) showed that VLDL isolated from hypertriglyceridemic (or type IV) subjects (HTG-VLDL) was a potent stimulator of ACAT. They showed that HTG-VLDL contains more apoE and more cholesterol/particle. However, a higher cholesterol content/HTG-VLDL particle, as compared with normal VLDL, cannot explain the discrepancy between their results and our results regarding the stimulation of ACAT activity. We observed that the cholesterol and apoE content (expressed as ratio cholesterol to triglycerides and apoE to apoB100, respectively) of the LPL-VLDL particles used in our study are in the same order of magnitude as that of the HTG-VLDL used by Evans *et al.* (34). Furthermore, expressing the amount of lipoprotein added in Fig. 7 as the amount of cholesterol added, instead of the amount of protein, did not considerably change the results shown.

Recently, Xu and Tabas (35, 36) have found that in mac-

rophages the cellular cholesterol level first have to reach a critical threshold of about 25% above the basal level, before ACAT activity is stimulated. If the same 25% increase in cellular cholesterol level is required in HepG2 cells in order to stimulate ACAT activity, our results indicate that, under the conditions applied, VLDL and LPL-VLDL do not increase the ACAT substrate pool enough for exerting an effect on the ACAT activity. Since the amount of uptake of VLDL and LPL-VLDL is comparable with the uptake of LDL (Fig. 1), also when based on the amount of cholesterol uptake (not shown), we conclude from our results that the ACAT substrate pool is supplied with lipoprotein-derived cholesterol only after the lipoproteins have been degraded. Hence, the cellular degradation of VLDL and LPL-VLDL is too inefficient to increase cellular cholesterol esterification.

A low degradation efficiency of VLDL and LPL-VLDL would also imply a relatively poor down-regulation of the LDL receptor activity upon incubation of cells with these lipoproteins. Epidemiological studies suggest that the down-regulation of the LDL receptor activity in the liver by VLDL and VLDL remnants depends, at least partly, on the polymorphism of apoE (37–39). Whether, besides affecting the binding of the lipoproteins to the receptor, apoE polymorphism also interferes with the efficiency of cellular degradation of VLDL or VLDL remnants, as a consequence of a retarded intracellular transport to the lysosomal compartment, is currently under investigation.

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