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 endocytic 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 lysosomal catabolism 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) is triglyceride-rich,

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‡ 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 lipase; LPL-VLDL, lipoprotein lipase-treated VLDL; PBS, phosphate-buffered saline; HTG, hypertriglyceridemic 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 remnants 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 (2–8).

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 endocytic 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. 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 catabolized about 1.6-fold more rapidly than LDL. In addition, we found that these lipoproteins fail to stimulate intracellular ACAT activity.

**EXPERIMENTAL PROCEDURES**

**Materials—**Fetal calf serum (FCS) and Dulbecco’s modified Eagle’s medium (DEM, cell culture medium) were obtained from Flow Laboratories (Irvine, United Kingdom). Human serum albumin (HSA) was obtained from Sigma. Na<sup>131</sup>I (specific activity 13.3 μCi/μl) was purchased from Amersham (Buckinghamshire, U.K.). Multicell culture wells were from Costar (Cambridge, MA). Proteinase K was purchased from Boehringer Mannheim (Mannheim, Germany). Defined in density 1.15 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 Rengwitz *et al.* (12). Lipoprotein lipase–treated VLDL (LPL-VLDL) were isolated by density gradient ultracentrifugation with LDL purified from bovine milk (13), essentially as described before (5). Binding of apo B to LDL 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 chloramine-T/periodate method, modified 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 <sup>131</sup>I-labeled lipoproteins were stored at 4°C for 14 days.

**26<sup>11</sup>I-labeled lipoproteins were stored at 4°C for 14 days. 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 <sup>131</sup>I-labeled lipoproteins were stored at 4°C for 14 days.**

**RESULTS**

**Time Course of Receptor-mediated Association and Degradation of LDL, LPL-treated VLDL, and VLDL from HepG2 Cells**—The time courses 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 LDL–VLDL and VLDL is only 50 and 20%, respectively, of that of LDL.

We reasoned that more information about the intracellular 26114 Retarded Intracellular Transport of VLDL
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 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-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. 4a) 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
been completed. When the presence of labeled particles in the incubation at the presence of acid phosphatase activity toward the late endosomal-lysosomal compartment has not been present in the light, early endosomal fractions. The rate of accumulation of the labeled lipoproteins in the high density fractions, which represent 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 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. 5a), after 15 min at 37 °C, almost all the radioactivity was found in the high density fraction, 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. 5b) and almost all VLDL (Fig. 5c) 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

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

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. 3. Initial endocytotic rate of LDL (a), LPL-VLDL (b), and VLDL (c) in HepG2 cells.](image-url)
Retarded Intracellular Transport of VLDL

**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 [3H]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 (Δ) 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.

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
The retarded intracellular routing of these particles might be the result of the multivalent 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 homeostasis, 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-

**Table 1**

<table>
<thead>
<tr>
<th>Lipoprotein degraded*</th>
<th>Lipoprotein present in the lysosomes*</th>
</tr>
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<tbody>
<tr>
<td>LDL</td>
<td>1.064 ± 0.063*</td>
</tr>
<tr>
<td>LPL-VLDL</td>
<td>1.61 ± 0.019</td>
</tr>
<tr>
<td>VLDL</td>
<td>1.59 ± 0.024</td>
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*Expressed in ng/mg cell protein/30 min.

#Expressed as radioactivity present in the high density fractions of Percoll gradients and expressed as ng/mg cell protein.

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.
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 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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