Recycling of Apolipoprotein E in Mouse Liver*

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Following the internalization of low density lipoprotein (LDL) by the LDL receptor within cells, both the lipid and the protein components of LDL are completely degraded within the lysosomes. Remnant lipoproteins are also internalized by cells via the LDL receptor as well as other receptors, but the events following the internalization of these complexes, which use apolipoprotein E (apoE) as their ligand for receptor capture, have not been defined. There is evidence that apoE-containing β-very low density lipoproteins follow differential intracellular routing depending on their size and apoE content and that apoE internalized with lipoproteins can be recycled by cultured hepatocytes and fibroblasts. In the present study, we addressed the question of apoE sparing or recycling as a physiologic phenomenon. Remnant lipoproteins (d < 1.019 g/ml) from normal mouse plasma were iodinated and injected into normal C57BL/6 mice. Livers were collected at 10, 30, 60, and 120 min after injection, and hepatic Golgi fractions were prepared for gel electrophoresis analysis. Golgi preparations were analyzed for galactosyltransferase enrichment (>40-fold above cell homogenate) and by appearance of the Golgi stacks and vesicles on electron microscopy. Iodinated apoE was consistently found in the Golgi fractions peaking at 10 min and disappearing by 2 h after injection. Although traces of apoB48 were present in the Golgi fractions, the apoE/apoB ratio in the Golgi was 50-fold higher compared with serum. Quantitatively similar results were obtained when the very low density lipoprotein remnants were injected into mice deficient in either apoE or the LDL receptor, indicating that the phenomenon of apoE recycling is not influenced by the production of endogenous apoE and is not dependent on the presence of LDL receptors. In addition, radioactive apoE in the Golgi fractions was part of d = 1.019–1.21 g/ml complexes, indicating an association of recycled apoE with either newly formed lipoproteins or the internalized complexes. These studies show that apoE recycling is a physiologic phenomenon in vivo and establish the presence of a unique pathway of intracellular processing of apoE-containing remnant lipoproteins.

The series of events following internalization of plasma low density lipoprotein (LDL)* has been dissected in detail by the classic studies of Goldstein and Brown (1, 2). The LDL particle, which contains apolipoprotein B100 (apoB100) as the sole protein component, is internalized as a lipoprotein-receptor complex within an endosomal compartment that subsequently fuses with lysosomes. Whereas the LDL receptor recycles back to the cell surface, the lipoprotein particle continues on its routing toward the lysosomal compartment where complete hydrolysis of apoB and the lipid components of the particle occurs (3). It is not known whether other lipoproteins follow the same intracellular pathways of LDL after internalization. Chylomicron and very low density lipoprotein (VLDL) remnants, for example, despite containing apoB utilize apoE as the ligand for receptor interactions (4, 5). In addition to binding to the LDL receptor, apoE can also interact with the LDL receptor-related protein (LRP) (6–8), as well as with heparan sulfate proteoglycans on the cell surface (9). Because of these multiple choices of cellular entry, it is possible that the fate of internalized apoE-containing lipoproteins may be different from that of LDL.

Previous studies have shown that the routing of β-VLDL, an apoE-containing remnant lipoprotein, is dependent upon size and possibly the apoE content of the lipoprotein. In two studies, Tabas et al. (10, 11) reported that smaller β-VLDL are routed to the perinuclear region of the mouse macrophage in a similar fashion to LDL, whereas larger β-VLDL remained closer to the plasma membrane. Although this phenomenon appeared to be mostly dependent on particle size, it is also possible that the higher apoE content of large β-VLDL was at least partially responsible for the different intracellular routing of this particle. Recent studies have shown that incubation of tissue culture cells with 125I-labeled lipoproteins results in the secretion of apoE-associated label after heparinase treatment (12, 13). In addition, following the injection of radiolabeled lipoproteins in rats, the contribution of exogenous apoB to the total apoB endosomal content was less than 10%, whereas that of exogenous apoE was higher than 60% (relative to total apoE) (14). These data would indicate that apoE internalized by cells may undergo complete degradation, and thus it can be hypothesized that some of this apoE may be utilized for resecretion.

Based on this information, we propose that apoE-containing lipoproteins undergo an intracellular destiny different from that of apoB-containing lipoproteins and that a portion of the apoE component of the lipoprotein is spared from degradation and routed through a secretory pathway that might involve the Golgi apparatus. A series of studies were performed in normal C57BL/6 mice and in mutant apoE and LDLR null mice to determine whether exogenously administered apoE could be found in the liver Golgi fraction at different time points after

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1 The abbreviations used are: LDL, low density lipoprotein; apo, apolipoprotein; VLDL, very LDL; LRP, LDL receptor-related protein; LDLR, LDL receptor.
intravenous injection of $^{125}$I-labeled remnant lipoproteins ($d < 1.019$ g/ml). Our studies show that apoE recycling is a physiologic phenomenon in the mouse liver in vivo. This phenomenon is evident in normal mice as well as in mice lacking apoE or the LDL receptor, suggesting that a different receptor, possibly the LRP, is involved in the selective retroendocytosis of apoE.

**MATERIALS AND METHODS**

**Preparation of Iodinated $d < 1.019$ Lipoproteins**—The $d < 1.019$ lipoproteins were prepared from Triton-treated mice as described previously (15). Briefly, in each experiment four donor C57BL/6 mice were treated with 10% tyloxapol (Sigma, 4 mg/kg body weight) via tail vein injection. Four hours following injection, mice were exsanguinated, and serum was separated by centrifugation. The $d < 1.019$ g/ml fraction was isolated by ultracentrifugation at 120,000 × g for 2.5 h on a Beckman TL-120 tabletop centrifuge. Iodination was carried out according to a modification of the McFarlane ICl method (16, 17). The iodinated $d < 1.019$ lipoproteins were dialyzed against 0.15 M NaCl. The protein concentration was determined by the Lowry procedure (18), and samples were diluted to 20 μg/ml. The integrity of the VLDL preparation was verified by electrophoresis on 4–12% SDS gels followed by exposure to film. The specific activities of the lipoprotein preparations ranged from 300 to 800 cpm/μg.

**Golgi Preparation**—Recipient mice were anesthetized with methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL), and 5 μg (200 μl) of radioactive fraction was injected into the jugular bulb. At various times following injection, blood was collected from the retro-orbital venous plexus. The mice were killed by cervical dislocation, and the liver was quickly removed, trimmed of excess fat and connective tissue, rinsed in distilled water, blotted, and weighed. Golgi apparatus-rich fractions were isolated from the liver according to a modification of the method of Swift et al. (19, 20). The livers were minced finely with scalpel blades and placed in homogenization buffer (0.1 M phosphate-buffered saline, pH 7.3, 0.05 M sucrose, 1% dextran, and 0.01 M MgCl$_2$). Pools of three livers were homogenized together for 15 s with a Polytron at setting 0.5 (model PT 10-35, Brinkmann Instruments). The homogenate was centrifuged in an SW 40 rotor at 4°C at 2600 rpm for 10 min and 9700 rpm for 30 min. The supernatant was discarded, and 1.7 ml of the homogenizing buffer was added to the pellet. The top one-third of the pellet was dislodged and layered on 8 ml of 1.2 M sucrose in an SW 40 centrifuge tube (5100 rpm for 10 min, 10,200 rpm for 10 min, and 25,600 rpm for 45 min). At the end of the centrifugation the white band at the interface was carefully removed, diluted with ice-cold distilled water, and pelleted in the SW 40 rotor at 10,000 rpm for 30 min. To analyze galactosyltransferase activity, the pellet was resuspended in 15 mM Tris, pH 7.4, 0.15 M NaCl and frozen in liquid nitrogen. To recover the nascent lipoproteins, the Golgi-enriched fraction was resuspended in 0.1 M Na$_2$CO$_3$, pH 11.0, and incubated on ice for 60 min. The membranes were pelleted by centrifugation in an SW 50.1 rotor at 45,000 rpm for 30 min. The supernatant was dialyzed against 0.15 M NaCl, 0.01% EDTA, pH 7.4, overnight. The density was raised to 1.019 g/ml using solid NaCl, and the $d < 1.019$ g/ml fraction was isolated by centrifugation in a Beckman Optima TLX tabletop ultracentrifuge at 120,000 rpm for 2.5 h. A $d < 1.019$ g/ml fraction was isolated from serum in a similar manner. The Golgi and serum lipoprotein fractions were stored in lyophilized form. A similar set of procedures was used in the experiment with rats. Because we cannot exclude the presence of minor endocytic contaminants, the Golgi preparations will be referred to as Golgi apparatus-enriched fractions throughout the article.

**SDS-Polyacrylamide Gel Electrophoresis**—SDS-polyacrylamide slab gels (3–20% gradient) were run as described by Swift et al. (20). Samples were solubilized in 0.06 M Tris-HCl, pH 6.8, 2.2% SDS, 10% glycerol, and 5% β-mercaptoethanol by heating at 100°C for 5 min and separated by gel electrophoresis. The gels were dried and exposed to film using DuPont Cronex Lightning Plus intensifying screens. Gels were exposed to film for up to 3 weeks. Densitometric analyses were performed on a MultiImage light cabinet equipped with AlphaImage 3.24 software (Alpha Innotech Corp., San Leandro, CA).

**Galactosyltransferase**—Galactosyltransferase activity was assayed using the method of Morre et al. (21) and modified to include ATP at the initial concentration of 1 mM to reduce nonspecific hydrolysis of the UDP galactose (22).

**Turnover Analyses**—At the specified time points after injection of the radiotracer, mice were killed and blood was collected. Perfusion of the carcass was done using 20 ml of cold saline injected at physiologic pressure from the tip of the heart after making an incision in the abdominal aorta below the renal artery level, and the perfusion continued until the liver was completely depleted of blood. The radioactivity in a 10-μl aliquot of plasma and the whole liver were measured in a γ-counter. Plasma clearance of the radiolabel was calculated assuming that the total plasma volume of a mouse is 3.5% of body weight and by dividing the total plasma counts at each time point by the injected amount (minus any losses during injection). Liver uptake at each time point was calculated as the ratio between liver counts and initial counts in plasma.
FIG. 2. Plasma clearance and liver uptake of the injected 125I-labeled d < 1.019 g/ml lipoproteins. Data points were calculated as described under “Materials and Methods.” Each data point is the average of three mice. Vertical bars represent standard error. Note that the y1 and y2 axes have different scales.

RESULTS

The aim of this study was to determine whether intact exogenous apolipoproteins could be identified in the Golgi apparatus-rich fractions of different strains of mice after injection of radiolabeled murine VLDL (d < 1.019 g/ml lipoproteins). The purity of mouse hepatic Golgi apparatus-enriched fractions was evaluated by both morphologic and biochemical criteria. By negative stain electron microscopy, the fractions exhibited morphologic characteristics consistent with the known features of the Golgi apparatus. Among the identifiable structures were cisternae, appearing as central plates or fenestrated plates continuous with an anastomosing tubular network containing secretory vesicles filled with lipoproteins (Fig. 1A). Intravascular particles, as well as particles inside plates, measured 40–70 nm, which is consistent with the size of VLDL. By thin section electron microscopy, the Golgi-enriched fractions consisted of parallel stacks of flattened cisternae along with secretory vesicles, some of which remained attached to the cisternae (Fig. 1B). Structures with characteristics of multivesicular bodies were not observed within our preparations of Golgi apparatus-enriched fractions (23). Biochemically, the purity of Golgi-rich fractions was assessed by the activity of galactosyltransferase, a Golgi-specific enzyme. Golgi-rich fractions were enriched in galactosyltransferase activity from 45- to 96-fold compared with the initial liver homogenates, not unlike values obtained using rat liver (19).

The fate of injected lipoproteins in a representative experiment is shown in Fig. 2. More than 70% of the label disappeared from plasma within 2 h after injection, whereas about 20% of the initial serum counts accumulated in the liver over the same period of time. The injected d < 1.019 g/ml lipoproteins were composed primarily of apoB100 and apoB48 and contained relatively smaller amounts of apoE (Fig. 3, A and B, lanes 1 and 2). On average, the radioactive label in the apoB proteins was 50-fold greater than in apoE. This preponderance of radioactive apoB was maintained in the plasma and VLDL fraction of the mice receiving the radiotracer, indicating that no selective loss or degradation of the injected particles was occurring. This ratio was dramatically altered in the luminal lipoproteins recovered from the Golgi apparatus-rich fractions of the liver of recipient mice where apoB100 was barely detectable, and apoB48 was consistently decreased compared with apoE (Fig. 4). Interestingly, this enrichment in apoE was most evident at 10 min postinjection, a time at which only a small portion of the radiolabeled material was taken up by the liver.

Selective sparing of apoE from the degradative pathway following lipoprotein internalization can best be shown by the appearance of internalized apoE in the secretory pathway. When C57BL/6 mice were injected with radiolabeled VLDL, the Golgi-rich fractions from pooled livers were selectively enriched in apoE (Fig. 3), an indication that a portion of the internalized apoE was carried back into the secretory pathway. Considering that the radioactivity distribution in the original VLDL preparation (Fig. 3A, lane 1) and in the plasma VLDL of mice after injection (Fig. 3A, lane 2) is more than 98% associated with apoB, it is apparent that the appearance of plasma apoE in the Golgi apparatus-enriched fractions represents a selective mechanism that excludes apoB100 and, to a lesser degree, apoB48. As determined in four separate experiments, the ratio apoE/apoB showed a 50-fold increase in the Golgi-rich fractions relative to the plasma compartment. The effect was time-dependent with the peak accumulation of labeled apoE being detected 10 min after injection (Fig. 3, A and B, lane 3) and progressively diminishing by 30 (Fig. 3A, lane 4) and 60 min (Fig. 3B, lane 4). No radioactivity in the Golgi-rich fraction was detected 120 min after injection of the radiotracer (data not shown). Plasma clearance of the injected tracer was only about 30% by the 10-min time point, and only about 13% of the initial
serum counts were detected in the liver (Fig. 2).

To determine whether the phenomenon of apoE recycling was affected by parameters such as the lack of endogenously produced apoE or the absence of the LDL receptor on the cell surface, we performed a similar series of experiments in genetically engineered mice carrying the homozygous disruption of either the apoE (24) or the LDL receptor gene (25). As shown in Fig. 5A (lanes 4 and 6), apoE was visible in the Golgi-enriched fractions of both groups of mice 10 min after the injection of 125I--VLDL. However, the time-dependent progression of internalized apoE in the Golgi apparatus-rich fraction of apoE(--/--) and LDLR(--/--) mice was different from that of normal mice with the peak apoE radioactivity being reached at 30 min after injection (Fig. 5B, lanes 2 and 4) rather than at 10 min. This was likely an effect of the retarded clearance of the radiotracer in mice lacking either apoE or the LDL receptor.

Studies were performed to examine apoE, which had reentered the secretory pathway, existed as a free protein or as part of a lipoprotein. Fig. 6 shows studies to determine the density distribution of radioactive Golgi apoE from livers of normal mice 10 min after injection of the tracer. Panel A shows that the radioactive apoE in the Golgi-rich fraction is primarily associated with a lipoprotein particle of density 1.006–1.210 g/ml, whereas some of the apoB48 could also be identified in the 1.006 g/ml region. Only traces of apoE were detected in the d < 1.006 g/ml and the lipoprotein-free bottom fractions. In Panel B, a similar experiment was performed to determine whether apoE in the Golgi apparatus-rich fraction would maintain the density distribution of the original VLDL preparation (d < 1.019 g/ml). As shown in lanes 3 and 4, a clear density shift occurs at the level of the Golgi-rich fraction, where all of the apoE is part of a complex with d > 1.019 g/ml.

Fig. 7 shows that the phenomenon of apoE enrichment in the liver Golgi is not limited to the mouse system but extends also to the rat, an established model of hepatic Golgi studies. The presence of internalized apoE in the Golgi-rich fraction of livers from rats 10 min after injection of radiolabeled rat VLDL was more dramatic than in the mouse system. ApoE was barely visible in the original preparation (not shown) and in the plasma of the injected animals (lane 1) and was selectively enriched in the Golgi-enriched fraction at the expenses of apoB100 and, to a lesser extent, apoB48.

**DISCUSSION**

In this study we show that the various apoprotein components of exogenously administered VLDL are processed differently by the mouse liver after internalization. The vast majority of the radioactivity in the 125I-labeled murine d < 1.019 g/ml lipoproteins was associated with apoB100 and apoB48, whereas only a small portion of the radioiodine was associated with apoE. The ratio apoE/apoB48 in the Golgi-rich fraction increased 10-fold relative to serum, and only traces of apoB100 were detected. The implications of such findings are as follows: 1) unlike LDL, components of the internalized VLDL are spared from complete intracellular degradation and appear in the secretory pathway; 2) apoB48-containing VLDL may have a different intracellular fate than apoB100-containing VLDL; and 3) apoE can recycle through the secretory pathway either free or associated with lipoproteins. It is important to notice that the phenomenon of apoE recycling to Golgi was also present in mice lacking either apoE or the LDL receptor, suggesting that the recycling of apoE is not affected by the production of endogenous apoE, which in liver does not appear to be increased by the common lipogenic stimuli (26, 27), and that the route of VLDL internalization followed by apoE recycling does not depend exclusively on the LDL receptor.

A possible argument against our interpretations is that the
enrichment of apoE within the Golgi-rich fraction simply reflects the prioritization of lipoprotein uptake by the liver with apoE-rich particles being selected more avidly and more rapidly for internalization than apoE-poor particles. This would be especially true if endosomal contamination was present in the Golgi preparations. Some Golgi apparatus-enriched fractions have been shown to be contaminated with endosomal vesicles (28). Elimination of this contaminant is crucial in assessing the true secretory products within the Golgi fraction. Our methodology for preparation of Golgi fractions from mouse liver was based on procedures developed in our laboratory (29) and subsequent modifications specifically to eliminate endosomal contamination, and apoB100 only represents a faint band in our Golgi-enriched preparations even 2 h after injection. Moreover, even if these results were partly because of endosomal contamination, the relevance of the observation would not be diminished, i.e. apoE undergoes an intracellular processing uniquely different from that of the apoB components of the same lipoprotein vehicle.

Although our studies do not define whether apoE recycles in free or lipoprotein-associated form, the data presented in Fig. 6 show that apoE in the Golgi-rich fractions is part of a lipoprotein complex of density smaller than the original radiolabeled VLDL. This could be due either to the association of recycling lipoprotein-free apoE with the nascent lipoproteins in the Golgi or to the recycling of apoE as part of smaller remnant particles. The presence of detectable levels of apoB48 in the Golgi-rich fraction allows us to speculate that the recycling particles may be small apoB48 remnants highly enriched in apoE. The finding that 125I-labeled apoB48 was isolated from the Golgi apparatus-enriched fraction floats in the same density range as 125I-labeled apoE is also compatible with this hypothesis and suggests the presence of a unique intracellular pathway in the liver that directs apoE-rich remnant particles through a recycling route, which may eventually lead to secretion of the particle as such or after repackaging.

The finding that in vivo recycling of apoE is not affected by the absence of the LDLR is a strong indication that additional receptors may be involved in this pathway. A possible candidate is the LDLR-related protein or LRP, which is abundantly expressed in the liver (6) and whose hepatic inactivation leads to dramatic accumulations of remnant lipoproteins in plasma (8, 31). LRP is uniquely responsive to apoE-containing lipopro-
teins, as was recently demonstrated in our laboratory, where mice lacking both LDLR and apoE were transplanted with wild-type bone marrow and showed no changes in lipoprotein levels despite high levels of macrophage-derived apoE in plasma (32). Additionally, this phenomenon may be related to direct lipoprotein uptake through heparan sulfate proteoglycans. Ji et al. (33) have presented evidence of a differential cellular accumulation of apoE isoforms in neuronal cells that is detectable in the absence of both LDLR and LRP but is eliminated by heparan sulfate proteoglycan inactivation. Finally, it is also possible that apoE recycling is dependent on the lipoprotein size, composition, and apoE content rather than on the kind of receptor involved in particle uptake.

The concept that internalized remnant may experience an intracellular fate different from that of LDL, although not of intuitive physiologic significance, is in line with previous studies of intracellular routing of internalized lipoproteins. Tabas et al. (10) demonstrated that the uptake of small remnants from macrophages in vitro was followed by a perinuclear distribution of the particles similar to that described for LDL. However, large remnant particles for the most part had a superficial distribution under the plasma membrane as well as a prolonged residence time within the cell (10). Although the authors reported that the size of the remnant particles was the main promoter of differential routing (11), it is possible that apoE content also plays a role in directing the particle to an alternate pathway, which may lead to particle resorption. This possibility has been suggested in recent in vitro studies, where incubation of either fibroblasts or HepG2 cells with 125I-labeled VLDL followed by heparinase treatment resulted in the appearance of 125I-labeled apoE in the media during the chase period (12, 13). Additional evidence for a unique role of internalized apoE is provided by a study in which cultured macrophages were incubated with model triglyceride-rich particles with or without apoE. Particles with apoE resulted in higher triglyceride degradation and higher cholesteryl ester synthesis than particles without apoE, indicating that apoE has effects on modulating intracellular lipid metabolism after lipoprotein internalization (34). The authors speculated that some apoE may be spared from lysosomal degradation to affect postlysosomal trafficking of lipid molecules to subcellular compartments such as the peroxisomes.

Two important issues that remain to be discussed are how and why apoE might recycle in vivo. A mechanistic insight is provided by transferrin (35). Transferrin, a major serum glycoprotein that transports iron into cells, binds to its receptor and is internalized in coated vesicles. Once in the endosome, the reduction in pH provokes the dissociation of iron from transferrin, but apotransferrin remains associated with its receptor and recycles back to the cell surface, thus escaping lysosomal degradation (36). From the invagination of coated pits to the formation of endosomes, the pathway of receptor-mediated endocytosis is common to all ligands studied. However, the fate of the receptor-ligand complex is determined in the endosome depending on whether the ligand can remain associated with the receptor at acidic pH. Ligands such as asialoglycoprotein and LDL dissociate from their receptors and are then routed to the lysosomal compartment (37). It is possible that, like transferrin, apoE has the ability to remain associated with its receptor(s) at the acidic pH of the endosome and to recycle back to the cell surface. Although it is not known whether apoE-containing lipoproteins remain associated to their receptors at low pH, if binding affinity for a recycling receptor plays any role in ligand retroendocytosis, the fact that apoE binds to its membrane receptor with a 23-fold higher affinity than apoB (38) may explain the differential routing of lipoproteins entering the cell through one or the other receptor.

It is evident from previous studies in our laboratory and other laboratories that only 10% of normal apoE levels is required to maintain cholesterol homeostasis under physiologic conditions when both the LDLR and the LRP are functioning normally (15, 39–41). In this situation, extrahepatic apoE will induce the clearance of lipoproteins without the need for the extra amount of ligand provided by the hepatic apoE secretion capture pathway (42–44). However, in situations where the LDLR does not function properly, additional apoE may be required in the sinusoidal space of Disse to facilitate the clearance of the accumulating remnant lipoproteins (32). ApoE retroendocytosis may represent a mechanism to ensure increased availability of apoE in the hepatic sinusoid. Additionally, recycling of apoE or apoE-rich lipoproteins may serve as a chaperone for proper targeting and repositioning of recycling LRP or other receptors to the cell surface (45). Because apoE is not structurally entangled within the lipoprotein particles, a system designed to reissue this functionally active molecule would be more efficient than simply targeting this ligand to the lysosomal compartment for proteolytic degradation.

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