Metabolism of Apolipoprotein E in Plasma High Density Lipoproteins from Normal and Cholesterol-fed Rats*

(Received for publication, January 18, 1982)

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High density lipoproteins of rat blood plasma were labeled in vitro with radiiodinated apolipoprotein E and biologically with [3H]cholesterol esters. These two components, present in high density lipoproteins separated from serum of normal or cholesterol-fed rats by molecular sieve chromatography, were removed slowly from perfused livers and the labeled apolipoprotein E was also removed slowly from the blood of intact rats. However, when labeled serum was subjected to ultracentrifugation at a density of 1.21 g/ml before the floating apolipoprotein E-labeled high density lipoproteins were separated by chromatography, the labeled protein was rapidly removed from the blood of intact rats by uptake into the liver. About one-half of the labeled apolipoprotein E associated with high density lipoproteins was dissociated during ultracentrifugation, but most of it reassociated with these lipoproteins when the floating lipoproteins were remixed with the sedimented serum proteins. The apolipoprotein E in such reassociated high density lipoproteins was removed from the blood of intact rats at the slow rate observed when the high density lipoproteins were separated chromatographically from whole serum. About 90% of the labeled apolipoprotein E in uncentrifuged or centrifuged high density lipoproteins was shown by affinity chromatography to be associated with particles containing apolipoprotein A-I. Rapid hepatic uptake of apolipoprotein E in centrifuged high density lipoproteins may result from an altered conformation of the apolipoprotein E on the particle surface.

Apolipoprotein E appears to be critically involved in the hepatic uptake and metabolism of partially degraded (remnant) lipoproteins of intestinal and hepatic origin (1-4). The concentration of apo-E in rat plasma is the highest reported among mammals fed cholesterol-poor diets. This apo-E is found mainly in high density lipoproteins (5, 6). We have developed a method to study the metabolism of apo-E in plasma lipoproteins of rats by a specific labeling technique and have shown that this protein is slowly removed from HDL when injected in either very low density lipoproteins or HDL (7). However, apo-E in VLDL was found to be removed rapidly, together with cholesteryl esters, from perfusates of isolated rat livers. The metabolism of apo-E in vivo appeared to result from rapid equilibration of apo-E between VLDL, in which it was rapidly metabolized, and HDL, in which it was metabolized slowly. 

* This work was supported by Grant HL-14237 from the United States Public Health Service, Specialized Center for Research in Atherosclerosis. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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The abbreviations used are: apo-E or A-I, apolipoprotein E or A-I; LDL, low density lipoproteins; VLDL, very low density lipoproteins; HDL, high density lipoproteins.

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EXPERIMENTAL PROCEDURES

**Treatment of Rats**—Male Sprague-Dawley rats weighing 300-350 g were maintained on standard Purina Rat Chow (Ralston Purina Co., St. Louis, MO) or fed a cholesterol-rich chow containing 5% lard, 1% cholesterol, 0.1% propylthiouracil, and 0.3% taurocholic acid for 2-3 weeks (6). Blood was collected from the animals as described earlier (7).

**Labeling Procedure and Separation of Lipoproteins**—Isolation and radiiodination of apo-E, incorporation of [3H]apo-E into lipoproteins of whole serum by incubation at 6 °C, and separation of labeled VLDL and HDL on columns of 8% agarose gel at 6 °C were performed as described (7). The separated lipoproteins were dialyzed for 2 h against 100 volumes of Krebs-Henseleit buffer (13) at 6 °C and were used in metabolic experiments within 3 h. In some experiments, apo-E labeled plasma was brought to a non-protein solvent density of 1.21 g/ml with solid KBr and subjected to ultracentrifugation at 12 °C for 45 h at 28,000 rpm (294 x 10^6 g min) in the 40.3 rotor of a Beckman preparative ultracentrifuge (14). The floating lipoproteins were obtained by tube slicing, brought to a volume of 10 ml with phosphate-buffered saline (7), and subjected to chromatography on 8% agarose gel to obtain the labeled HDL. Alternatively, the 40.3 rotor tubes, after centrifugation, were gently mixed at room temperature for 15 min and the entire contents of the tube were brought to a volume of 10 ml as above and subjected to chromatography. To label cholesteryl esters of VLDL and HDL, a solution of [3H]cholesterol, stabilized by bovine serum albumin (15), was injected into donor rats 8 h before they were bled, under diethyl ether anesthesia, from the abdominal aorta. 

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Metabolic Studies—Labeled lipoproteins containing an amount of cholesterol equivalent to less than 5% of that in the corresponding lipoprotein in the blood of the recipient rat were injected through the femoral vein of animals anesthetized with diethyl ether and blood samples were collected from a tail vein (7). In some experiments, samples of liver were obtained at the end of the experiment for analysis of apo-A-I (7). Isolated livers were perfused in a recirculating system with Krebs-Henseleit/glucose buffer containing 18% rat erythrocytes (7). For experiments with labeled HDL, the perfusate volume was 60 ml and samples of 2 ml were taken for analysis at the indicated intervals. For experiments with labeled VLDL, the perfusate volume was 35 ml and sample volumes were 1 ml. At the end of perfusions, livers were flushed with 60 ml of fresh perfusate and several samples were taken for analysis. Samples of blood, perfusate, and liver were subjected to analysis of trichloroacetic acid-soluble and insoluble 125I- or 3H cholesterol esters exactly as described (7).

Chemical Analyses—Protein was determined by a modified procedure of Lowry with bovine serum albumin as standard (16). Apo-protein E was measured by specific radioimmunoassay (5). Cholesterol in serum and in lipoprotein fractions separated by sequential preparative ultracentrifugation (14), or molecular sieve chromatography, was measured by a modification of a fluorometric enzymatic method (17).

Affinity Chromatography of HDL—An amount of chromatographically separated HDL, equivalent to that contained in 1 ml of plasma, was dialedyzed against 0.15 M NaCl, 0.02% sodium azide, and 0.04% diosodium EDTA, pH 7.4, and applied to an affinity column (18) (1 x 10 cm) packed with monospecific rat anti-apo-A-I. The column was maintained at 6 °C. Fractions containing unbound lipoprotein were collected for analysis of apo-E. The capacity of this column for apo-A-I was ~3 mg. When 1 mg of isolated apo-E was applied to the column, no detectable protein was retained.

RESULTS

Uptake and Catabolism of Chromatographically Separated 125I-Apo-E HDL by the Perfused Liver—HDL from chow-fed rats, labeled biologically with [3H]cholesterol and in vitro with 125I-apo-E, were separated from serum on 6% agarose columns and dialyzed against Krebs-Henseleit buffer and then added to perfusates of isolated rat livers. Concentration of 125I in cholesteryl esters and trichloroacetic acid (TCA)-soluble 125I in perfusates was measured in samples taken at the times indicated. The mass of total cholesterol added to the perfusate was 0.87 ± 0.09 mg. Results are mean values ± S.D. for four experiments.

FIG. 1. Uptake and metabolism of doubly labeled HDL by isolated, perfused rat livers. The serum lipoproteins were labeled biologically with [3H]cholesterol and 125I-apo-E (7) and the HDL, separated on columns of 6% agarose gel, were dialyzed for 1-2 h against Krebs-Henseleit buffer and then added to perfusates of isolated rat livers. Concentration of 125I in cholesteryl esters and trichloroacetic acid (TCA)-soluble 125I in perfusates was measured in samples taken at the times indicated. The mass of total cholesterol added to the perfusate was 0.87 ± 0.09 mg. Results are mean values ± S.D. for four experiments.

FIG. 2. Uptake of [3H]cholesterol esters in HDL from perfusates by isolated perfused rat livers. Two fractions of HDL separated from serum on columns of 6% agarose gel were used: a high molecular weight fraction containing 0.15 ± 0.05 mg of total cholesterol and 0.7 ± 0.04 mg of apo-E, obtained from the leading portion of the HDL elution profile from the column (O—O) and a low molecular weight fraction containing 0.85 ± 0.05 mg of total cholesterol and 0.07 ± 0.04 mg of apo-E, obtained from the trailing portion of the HDL elution profile (■—■). The middle portion of the elution profile was discarded. Results are mean values ± S.D. for three experiments.

was subjected to chromatography on 6% agarose gel. The infranatant fraction contained 47.7 ± 2.3% of the 125I-apo-E (n = 4), which represented about one-half of the recovered 125I (total recovery was 94.1 ± 1.7%). When the original serum was subjected to chromatography on 6% agarose gel, only 13.6 ± 1.6% of the 125I-apo-E was recovered in the region expected for unbound apo-E, indicating that about 35% of the 125I-apo-E was dissociated from lipoproteins during ultracentrifugation. In Fig. 3A, elution profiles of 125I-apo-E-labeled serum and the labeled lipoproteins separated by ultracentrifugation at 1.21 g/ml are compared. It is evident that most of the 125I-
The extent to which ultracentrifugation affected the association of apo-E and apo-A-I, the other major protein component of rat HDL, on HDL particles was evaluated by chromatography on 6% agarose columns and then passed through an anti-A-I affinity column. The fraction of apo-E that was not adsorbed to the column was taken as an estimate of the unassociated apo-E. As shown in Table I, only about 10% of the apo-E appeared to be in HDL particles that did not contain apo-A-I, both in native serum and after ultracentrifugation.

\[125\text{I-apo-E HDL, isolated by gel chromatography of either whole serum or the } d < 1.21 \text{ g/ml fraction obtained by ultracentrifugation, was injected into intact rats. As shown in Fig. 4A, ultracentrifugation caused a dramatic increase in the rate of removal of the } 125\text{I-apo-E from the blood. In two experiments, 51.2\% and 58.7\% of the injected } 125\text{I-apo-E in ultracentrifuged HDL was found in the liver 15 min after injection of the ultracentrifuged HDL. In the experiments shown in Fig. 4A, the HDL were injected into rats approximately 3 days after the serum was labeled with apo-E. Comparable results were obtained when HDL prepared from uncentrifuged serum were injected immediately after elution from agarose columns.}

\[\text{TABLE I}

\text{Affinity chromatography of apo-E-containing HDL from cholesterol-fed rats}

\begin{tabular}{|c|c|c|}
\hline
Preparation of HDL & Apo-E applied to column & Apo-E not bound to antibody \\
\hline
Chromatographically separated from serum & 107 ± 12 & 9 ± 4 \\
Chromatographically separated from ultracentrifuged lipoproteins & 58 ± 9 & 6 ± 3 \\
\hline
\end{tabular}

Fig. 3. Molecular sieve chromatography of 125I-apo-E-labeled lipoproteins from rats fed standard chow. A, separation on a column of 6% agarose gel of 125I-apo-E in 10 ml of serum from rats fasted for 24 h (A—A) and in lipoproteins separated from serum by ultracentrifugation at a density of 1.21 g/ml (Δ—Δ). The first peak represents 125I-apo-E in VLDL and the second peak that in HDL. Unassociated apo-E is represented by the third peak (not seen in separated lipoproteins). The absorbance of serum lipoproteins and proteins at 280 nm is also shown (O—O). B, separation on a column of 6% agarose gel of 125I-apo-E in 10 ml of serum (A—A) and in serum that had been subjected to ultracentrifugation at a density of 1.21 g/ml to separate lipoproteins, which were then remixed with the serum proteins that had sedimented to the bottom of the centrifuge tube (Δ—Δ).

apo-E dissociated by ultracentrifugation was derived from the HDL. In four such experiments, 10.2 ± 4.5% of the 125I-apo-E added to the serum was lost from VLDL, whereas 48.1 ± 9.1% was lost from HDL. In spite of this large loss of 125I-apo-E from HDL, the elution volume of the HDL-associated apo-E was unaltered by ultracentrifugation. In one experiment, in which unlabeled serum and its 1.21 g/ml supernatant fraction were compared, elution of apo-E from the 6% agarose columns was measured by radioimmunoassay. Losses of apo-E mass from VLDL and HDL during ultracentrifugation were comparable to those observed with 125I-apo-E (not shown).

The extent to which ultracentrifugation affected the association of apo-E and apo-A-I, the other major protein component of rat HDL, on HDL particles was evaluated by immunoadsorption. The HDL fraction of samples of rat serum and of the 1.21 g/ml supernatant fraction of serum were obtained by chromatography on 6% agarose columns and then passed through an anti-A-I affinity column. The fraction of apo-E that was not adsorbed to the column was taken as an estimate of the unassociated apo-E. As shown in Table I, only about 10% of the apo-E appeared to be in HDL particles that did not contain apo-A-I, both in native serum and after ultracentrifugation.

Fig. 4. Removal of 125I-apo-E HDL from blood plasma. A, removal from blood plasma of intact rats of 125I-apo-E HDL separated on a column of 6% agarose gel from lipoproteins obtained from serum by ultracentrifugation at a density of 1.21 g/ml for 48 h and dialyzed overnight against Krebs-Henseleit buffer (O—O), or separated directly from uncentrifuged serum and stored at 5 °C for 72 h (C—C). Results are mean values ± S.D. for four experiments. B, removal from blood plasma of intact rats of 125I-apo-E HDL, prepared from lipoproteins that were subjected to ultracentrifugation as in A and then recombined with the sedimented serum proteins, as described in the legend to Fig. 3B. Results are mean values ± S.D. for three experiments.
As shown in Fig. 3B, the \(^{125}\text{I}-\text{apo-E}\) dissociated from HDL during ultracentrifugation at a density of 1.21 g/ml was found to reassociate with the lipoprotein when the supernatant and infranatant fractions were remixed. After such recombination of \(^{125}\text{I}-\text{apo-E}\), only 20.2 ± 2.1% of the labeled protein was unassociated with VLDL and HDL separated on 6% agarose columns, as compared with 12.7 ± 3.7% when the same samples of uncentrifuged serum were applied to the columns. Comparable results were obtained for reassociation of apo-E mass, as determined by radioimmunoassay in other experiments.

**TABLE II**

<table>
<thead>
<tr>
<th>Concentration of cholesterol in plasma lipoproteins of cholesterol-fed rats</th>
<th>mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction (density in g/ml)</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>&lt;1.006</td>
<td>130.6 ± 10.8*</td>
</tr>
<tr>
<td>1.006–1.019</td>
<td>76.0 ± 11.8</td>
</tr>
<tr>
<td>1.019–1.063</td>
<td>45.1 ± 6.3</td>
</tr>
<tr>
<td>&gt;1.063</td>
<td>15.5 ± 4.6</td>
</tr>
</tbody>
</table>

* Mean ± S.D. (total cholesterol and apo-E levels in unfractionated serum were 295.7 ± 6.9 and 41.9 ± 5.1 mg/dl, respectively).

FIG. 5. Distribution of cholesterol and apo-E in lipoproteins of serum from rats fed standard or cholesterol-rich diets. A, separation on a column of 6% agarose gel of total cholesterol (O—O) and apo-E (A—A) in 10 ml of serum from cholesterol-fed rats fasted for 24 h. The two peaks represent (from left) VLDL and HDL. The absorbance of serum lipoproteins and proteins is also shown (O—O). B, separation on a column of 6% agarose gel of cholesterol and apo-E in 10 ml of serum from a rat fed regular chow and fasted for 24 h. Symbols are as in A.
apo-E, added to serum from hypercholesterolemic rats, was comparable to that of apo-E mass; 15.7 ± 2.4% of the $^{125}$I-apo-E remained unassociated with VLDL or HDL (n = 4). As in normal rats (7), the $^{125}$I-apo-E was stable as determined by rechromatography of the isolated fractions, and the labeled apo-E readily exchanged between VLDL and HDL, both in vitro and after injection into intact rats (not shown). The rate of removal of $[^3]H$cholesterol esters in HDL from hypercholesterolemic rats, during perfusion through livers of chow-fed rats (Fig. 6), was similar to that observed with HDL from chow-fed rats (Fig. 1), as was the rate of production of trichloroacetic acid-soluble $^{125}$I-apo-E. In these experiments with HDL from cholesterol-fed rats, 17.2 ± 5.7% of the $[^3]H$cholesterol esters and 12.4 ± 3.5% of the $^{125}$I-apo-E remaining in the perfusate after 4 h was associated with VLDL. Recovery of $^{125}$I and $[^3]H$ in liver and perfusate at the end of the experiments was 96.3 ± 2.5 and 92.7 ± 5.1%, respectively.

Similar experiments were performed with doubly labeled VLDL from hypercholesterolemic rats (Fig. 7). The rates of removal of $^{125}$I-apo-E and $[^3]H$cholesterol esters from the perfusate were indistinguishable, but somewhat more rapid than observed with VLDL from chow-fed rats (7). At the end of the 21-min perfusion period, 11.2 ± 2.0% of the $[^3]H$cholesterol esters and 17.9 ± 7.2% of the $^{125}$I-apo-E remaining in the perfusate after 4 h was associated with VLDL. Recovery of $^{125}$I and $[^3]H$ in liver and perfusate at the end of the experiments was 96.3 ± 2.5 and 92.7 ± 5.1%, respectively.

In confirmation of our earlier research in which $^{125}$I-apo-E HDL from chow-fed rats was injected into intact rats (7), we found that the labeled protein was removed slowly from perfusates of isolated rat livers and then catabolized completely. $[^3]H$Cholesterol esters of these chromatographically separated HDL were taken up from the perfusate at a lower rate than $^{125}$I-apo-E, but labeled cholesterol esters in the larger, apo-E-rich HDL particles were removed more rapidly than those in the smaller, apo-E-poor ones. It seems likely, therefore, that the two types of particles are removed by the liver at a similar rate.

The fractional rates of removal of cholesterol esters and apo-E of chromatographically separated HDL by perfused livers were more rapid than those observed earlier with HDL isolated from rat blood plasma between densities of 1.08S and 1.21 g/ml, which were labeled endogenously in the cholesteryl ester moiety with $[^3]H$ or exogenously in the protein moiety with $^{125}$I (19). In those experiments, the major labeled protein was apoprotein A-I and much higher concentrations of HDL were routinely added to the perfusates. However, even when amounts of HDL comparable to those used in the present experiments were added, the fractional rates of removal of $^{125}$I did not approach those observed in the present studies. The reasons for the more rapid removal of the chromatographically separated HDL by the perfused liver are unclear, but it is possible that $^{125}$I-apo-E exchanged with unlabeled apo-E secreted from the liver in VLDL, and was then taken up by the liver during endocytosis of these VLDL (1). In our previous studies in which $^{125}$I-apo-E and $^{125}$I-apo-A-I HDL were injected into intact rats, removal of $^{125}$I-apo-E slightly exceeded that of $^{125}$I-apo-A-I (7), but the rates for both were similar to those of ultracentrifugally separated, radioiodinated HDL (19).

As expected from earlier work (6), apo-E-containing HDL from cholesterol-fed rats (HDLc) were larger than those from rats fed regular chow. The removal of $[^3]H$cholesterol esters and $^{125}$I-apo-E from these HDL by perfused livers did not exceed that of such HDL obtained from rats fed regular chow. Others have obtained variable results in their studies of the metabolism of HDL containing apo-E. The apo-E of the HDL, that has been obtained from blood plasma of cholesterol-fed dogs by a combination of ultracentrifugation and preparative electrophoresis is rapidly removed from the blood by the liver (9, 10). Apo-E-containing HDL from chow-fed rats, obtained from ultracentrifuged HDL by chromatography on heparin-Sepharose (11), is also rapidly taken up from the blood by the liver at a rate greater than that of apo-E-containing HDL from cholesterol-fed rats (11).

$^{a}$ Given a pool size of apo-E in HDL of 2 mg (see Ref. 12) and a fractional catabolic rate of 0.11 h$^{-1}$ (see Ref. 7), the production rate of HDL apo-E is 0.22 mg h$^{-1}$, as compared with a production in perfused livers of about 0.5 mg h$^{-1}$ (27).
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liver. By contrast, the apo-E-containing HDL obtained by preparative electrophoresis of the 1.02-1.063 g/ml ultracentrifugal fraction of plasma of rats fed cholesterol-rich chow is removed considerably more slowly from blood plasma of rats than canine apo-E HDL (9).

All HDL obtained by ultracentrifugation have lost a portion of their component apo-E. Our present experiments show apo-E in ultracentrifuged HDL, whether obtained from rats fed regular chow or cholesterol-rich chow, to be rapidly taken up from the blood by the liver, even though the apo-E-containing HDL particles contained at least 1 molecule of apo-A-I. Of considerable interest was the further observation that the apo-E that was dissociated from these particles by ultracentrifugation largely reassocited with the HDL upon simple mixing of the separated components, as shown by the profile of the eluted apo-E when the “recombined” plasma was subjected to gel chromatography. These results indicate that loss of apo-E during ultracentrifugation does not alter apo-E-containing HDL irreversibly. They also provide strong evidence that partial loss of apo-E or some other dissociable component rather than ultracentrifugation per se alters the hepatic uptake of apo-E-containing HDL particles, presumably by receptor-dependent processes (20-23). The ultracentrifugally modified HDL are distributed over the same range of size as unmodified HDL containing apo-E (see Fig. 3). This suggests that these particles remain “intact,” but with an altered conformation of surface components.

Apo-E in canine apo-E-HDLs, obtained by ultracentrifugation and preparative electrophoresis, is taken up by the liver in a process that closely resembles that of chylomicron remnants (8). In these particles, apo-E is virtually the sole protein component (8). By contrast, most of the apo-E-containing HDL in our rats fed regular chow contained at least 1 molecule of apo-A-I, whether obtained directly from serum or from ultracentrifuged lipoproteins (see Table I). Thus, the presence of other proteins in HDL particles that contain apo-E does not necessarily impair hepatic uptake. Our present studies, as well as earlier ones (1, 2, 4), also suggest that recognition of apo-E by an hepatic receptor or receptors does not depend solely upon the number of apo-E molecules present on a lipoprotein particle, or upon a high concentration of apo-E at the particle surface. Discooidal complexes of rat apo-E with lecithin, that contain about 8 molecules of apo-E per particle, and apo-E-HDLs bind to liver membrane preparations from estradiol-treated rats with similarly high affinity (21). These membrane preparations express a high concentration of binding sites whose properties closely resemble those of the “LDL receptor” of cultured human fibroblasts. However, the uptake of ultracentrifugally modified HDL containing apo-E by the liver is considerably more efficient than that of such discoidal complexes (4). Interaction of apo-E with at least one hepatic receptor, perhaps a specific “remnant” receptor (22, 23) rather than the “LDL receptor” that recognizes both apo-B and apo-E, may be favored in ultracentrifugally isolated HDL containing apo-E by reduction of the concentration of the protein at the particle surface. The recognition site, which seems likely to contain a high concentration of positively charged amino acids (24, 25), evidently is better exposed under conditions of lower concentration of apo-E on the particle surface, possibly owing to reduction of protein-protein interactions. By contrast, VLDL from cholesterol-fed rats, rich in cholesteryl esters and apo-E, were found to be removed more rapidly from liver perfusates than VLDL from rats fed regular chow. These results are consistent with earlier studies in which the proportion of apo-E in VLDL-protein was altered by mixing VLDL with VLDL-free serum in vitro (1).

Although our results suggest that apo-E-containing HDL do not constitute a unique vehicle in the rat for transport to the liver of cholesteryl esters synthesized by lecithin-cholesterol acyltransferase, they do not exclude an important role for such HDL in cholesteryl ester transport. As suggested earlier (19), arachidonate-rich cholesteryl esters of HDL may participate in the transport of cholesteryl esters to extrahepatic tissues. In view of the low concentration of LDL and the very limited cholesteryl ester transfer activity in rat plasma (26), those HDL that contain apo-E may be a major vehicle for the transport of cholesterol to cells containing active “LDL receptors.” The amount of such HDL in the rat may be sufficient to provide an effective substitute for LDL in the delivery of cholesterol to the liver as well as extrahepatic tissues.

Acknowledgment—We are grateful to Luuko Gao for use of the anti-A-I affinity column.

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