The two-receptor model of lipoprotein clearance: Tests of the hypothesis in "knockout" mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins

( hypercholesterolemia/chylomicrons/very low density lipoproteins/atherosclerosis/gene targeting)

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ABSTRACT Apolipoprotein E (apoE) is hypothesized to mediate lipoprotein clearance by binding to two receptors: (i) the low density lipoprotein receptor (LDLR) and (ii) a chylomicron remnant receptor. To test this hypothesis, we have compared plasma lipoproteins in mice that are homozygous for targeted disruptions of the genes for apoE [apoE(-/-)], the LDLR [LDLR(-/-)], and both molecules [apoE(-/-); LDLR(-/-)]. On a normal chow diet, apoE(-/-) mice had higher mean plasma cholesterol levels than LDLR(-/-) mice (579 vs. 268 mg/dl). Cholesterol levels in the apoE(-/-); LDLR(-/-) mice were not significantly different from those in the apoE(-/-) mice. LDLR(-/-) mice had a relatively isolated elevation in plasma LDL, whereas apoE(-/-) mice had a marked increase in larger lipoproteins corresponding to very low density lipoproteins and chylomicron remnants. The lipoprotein pattern in apoE(-/-); LDLR(-/-) mice resembled that of apoE(-/-) mice. The LDLR(-/-) mice had a marked elevation in apoB-100 and a modest increase in apoB-48. In contrast, the apoE(-/-) mice had a marked elevation in apoB-48 but not in apoB-100. The LDLR(-/-); apoE(-/-) double homozygotes had marked elevations of both apolipoproteins. The observation that apoB-48 increases more dramatically with apoE deficiency than with LDLR deficiency supports the notion that apoE binds to a second receptor in addition to the LDLR. This conclusion is also supported by the observation that superimposition of a LDLR deficiency onto an apoE deficiency [apoE(-/-); LDLR(-/-) double homozygotes] does not increase hypercholesterolemia beyond the level observed with apoE deficiency alone.

Genetic blocks in the catabolism of chylomicron remnants are important causes of hypercholesterolemia and atherosclerosis in humans. The prototypic diseases are familial hypercholesterolemia, caused by a defect in the low density lipoprotein receptor (LDLR) (1), and familial type III hyperlipoproteinemia, caused by a defect in one of the ligands for this receptor, apolipoprotein E (apoE) (2).

To create murine models for these diseases, the laboratories of Maeda (3) and Breslow (4) used the technique of homologous recombination in embryonic stem cells to produce strains of mice lacking apoE. Ishibashi et al. (5, 6) used the same technology to inactivate the gene for the LDLR. Homozygous apoE(-/-) mice and LDLR(-/-) mice exhibited hypercholesterolemia, but the severity and manifestations differed markedly. On a normal chow diet, the apoE(-/-) mice had much more profound hypercholesterolemia (plasma cholesterol, ~600 mg/dl) (3, 4) as compared with the LDLR(-/-) mice (~250 mg/dl) (5). Moreover, the distribution of the cholesterol differed. In the apoE(-/-) mice, cholesterol accumulated primarily in large lipoprotein particles with a size corresponding to chylomicron remnants, very low density lipoproteins (VLDLs), and intermediate density lipoproteins (IDLs) (3, 4). In contrast, the LDLR(-/-) mice had a much more selective elevation in LDL (5, 6). The apoE(-/-) mice (3, 4) and the LDLR(-/-) mice (5, 6) both exhibited a profound hyperresponsiveness to dietary cholesterol, with plasma cholesterol levels rising above 2000 mg/dl.

The differences between the two mutant strains of mice are consistent with current models of the differing roles of apoE and the LDLR in the metabolism of cholesterol-rich lipoproteins (1, 2). These lipoproteins enter the circulation either from the intestine (chylomicrons) or the liver (VLDL). They are partially metabolized by lipoprotein lipase, generating remnant particles known as chylomicron remnants and IDLs, respectively, that are cleared by binding to receptors in the liver.

The bulk of IDL particles are cleared by the liver via the LDLR. When the LDLR is defective, as in familial hypercholesterolemia homozygotes or WHHL rabbits, the IDLs remain in the circulation where they are converted to LDLs (1, 7, 8). IDLs contain two apolipoproteins capable of binding to the LDLR. These are apoE, which binds with high affinity, and apoB-100, which binds with lower affinity (9, 10). Genetic evidence indicates that apoE is more important in the clearance of IDLs. Thus, in humans with defective apoE, IDLs accumulate in the circulation despite the normal content of apoB-100 (2). In contrast, humans with defective apoB-100 accumulate LDL but not IDL (11).

The clearance of chylomicron remnants is more complex. These particles contain apoB-48, which lacks the C-terminal half of apoB-100 and cannot bind to LDLRs. Hence, clearance of chylomicron remnants is dependent almost entirely on apoE (10). Under normal circumstances, many chylomicron remnants are cleared by the LDLR. However, chylomicron remnants do not accumulate in familial hypercholesterolemia homozygotes (1, 12) or WHHL rabbits (13), indicating that the liver has a backup system capable of clearing these particles when the LDLR is absent. This backup system must also rely on apoE since chylomicron remnants accumulate in type III hyperlipoproteinemic patients with defective apoE (2). Recent evidence suggests that this backup clearance is mediated by the LDLR-related protein (LRP), which binds apoE-enriched remnant particles (14, 15). LRP may act in concert with proteoglycans on the surface of hepatocytes (16).

Abbreviations: apoE, apolipoprotein E; FPLC, fast performance liquid chromatography; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LDLR, LDL receptor; LRP, LDLR-related protein; VLDL, very low density lipoprotein.
Lipoprotein metabolism in mice differs importantly from that in humans and rabbits in that 70% of the VLDL particles produced in the mouse liver contain apoB-48 rather than apoB-100 (17). Thus, most mouse VLDLs are metabolized like chylomicron remnants and are not converted to LDLs.

The availability of apoE(-/-) and LDLR(-/-) mice permits additional tests of the two-receptor model of lipoprotein clearance. Inasmuch as apoE binds to LRP as well as to the LDLR (18), the level of plasma apoB-48 should be higher in apoE-deficient mice than in LDLR-deficient mice. In contrast, LDLR-deficient mice should have an isolated elevation in apoB-100. In an animal with a simultaneous deficiency of apoE and the LDLR, apoB-48 and apoB-100 should both accumulate.

In the current study, we have begun to test these predictions by producing double-homozygous mice that are deficient in apoE as well as in LDLRs [apoE(-/-); LDLR(-/-)]. To avoid potential problems with interlaboratory differences, all mice were raised in the same environment in Dallas and were fed the same diet. The results provide further support for the two-receptor model of lipoprotein clearance.

EXPERIMENTAL PROCEDURES

Materials and Methods. Blood was obtained from the retroorbital plexus as described (5). Plasma cholesterol and triglycerides were determined enzymatically with assay kits obtained from Boehringer Mannheim and Sigma, respectively. Plasma lipoproteins were analyzed by fast performance liquid chromatography (FPLC) gel filtration as described (6). For apolipoprotein analysis, the total lipoprotein fraction (d < 1.215 g/mL) was dialedyzed against a solution containing 0.15 M NaCl, 1 mM sodium EDTA, and 1 mM phenylmethylsulfonyl fluoride at pH 7.4 and delipidated with ethanol/ether (1:1). Apolipoproteins were boiled for 3 min in SDS sample buffer containing 5% (vol/vol) 2-mercaptoethanol/8 M urea and incubated at 37°C for 1 h prior to SDS/PAGE (5). Total cholesterol content of liver was measured as described (19) except that the blood remaining in the liver was removed by flushing the portal vein with 0.15 M NaCl before taking samples.

Mice. LDLR "knockout" mice, LDLR(-/-) (5), and apoE knockout mice, apoE(-/-) (5), were created by targeted gene disruption as described in the indicated references. To obtain knockout mice that are homozygous for disruption of the LDLR and apoE loci, male apoE(+) mice were mated to female LDLR(-/-) mice. The resulting apoE(+/-); LDLR(-/-) mice were identified by Southern blot analysis and bred to each other to produce apoE(-/-); LDLR(-/-) mice. Experiments were performed with these mice or those with the same genotype from the next generation obtained by breeding apoE(-/-); LDLR(-/-) with each other. All mice used in experiments are hybrids between C57BL/6J and 129Sv strains. Mice were maintained on 12-h dark/12-h light cycles and were allowed access to food and water ad libitum.

Diets. Two diets were used: (i) a normal chow diet (Teklad 4% mouse/rat diet 7001 from Harlan Teklad Premier Laboratory Diets, Madison, WI) that contained 4% (wt/wt) animal fat and <0.04% (wt/wt) cholesterol; (ii) a 1.25% cholesterol/atherogenic diet (3 parts of the normal chow diet mixed with 1 part of a cholesterol, cocoa butter, casein, sodium cholate diet (TD78399 from Harlan Teklad). The final diet contained 1.25% cholesterol, 7.5% (wt/wt) cocoa butter, 7.5% casein, and 0.5% (wt/wt) sodium cholate.

RESULTS

Fig. 1 compares the total plasma cholesterol levels in nonfasting mice of four different genotypes, all of which were born and raised in a single environment in Dallas and fed a normal chow diet. As reported previously (3-5), plasma cholesterol levels in the apoE(-/-) mice (mean, 579 mg/dl) were much higher than those in the LDLR(-/-) mice (mean, 268 mg/dl). Cholesterol levels in the double homozygotes [apoE(-/-); LDLR(-/-)] were not significantly different from the levels in the apoE(-/-) mice (620 mg/dl). The plasma triglyceride levels in a random sample of nonfasting mice from the four genotypes (8–17 animals per group) on a chow diet were as follows: wild-type mice (89 ± 6 mg/dl; mean ± SE), LDLR(-/-) (149 ± 17), apoE(-/-) (180 ± 32), and apoE(-/-); LDLR(-/-) (176 ± 48).

When wild-type mice were fed a diet containing 1.25% cholesterol plus cholic acid, saturated fat, and casein (1.25% cholesterol/atherogenic diet), the plasma cholesterol level increased only slightly (Fig. 2A). In the LDLR(-/-) mice, the plasma cholesterol increased dramatically within 2 weeks, and this was sustained for 8 weeks. The apoE(-/-) mice also showed a dramatic response to cholesterol feeding, as did the apoE(-/-); LDLR(-/-) double homozygotes.

Analysis of plasma lipoproteins by FPLC gel filtration revealed that the LDLR(-/-) mice on a chow diet had the expected peak in the size range of LDL (Fig. 2B). When these animals were fed the 1.25% cholesterol/atherogenic diet, there was a marked accumulation of larger lipoproteins, whose size corresponded to chylomicron remnants, VLDLs, and IDLs. In the apoE(-/-) mice on a normal chow diet, most of the cholesterol was contained in lipoproteins corresponding to chylomicron remnants, VLDLs, and IDLs. The 1.25% cholesterol/atherogenic diet elevated all of these lipoproteins, but it preserved the relative size distribution. The apoE(-/-); LDLR(-/-) mice on a normal chow diet showed an elevation in chylomicron remnants, VLDLs, and IDLs and a separate peak indicating an elevation of lipoproteins in the size range of LDLs. When these animals were fed the 1.25% cholesterol/atherogenic diet, all of these lipoproteins were increased in amount. For purposes of quantification, we arbitrarily pooled fractions from the FPLC and classified them as chylomicron/VLDL, IDL/LDL, and high density lipoprotein. The largest increase in cholesterol content was seen in the chylomicron/VLDL fraction of all three
mutant genotypes fed the cholesterol/atherogenic diet (Table 1). A lesser but still marked increase occurred in the IDL/LDL fraction of all three genotypes (Table 1).

Fig. 3 shows the apolipoprotein content of the total pooled lipoprotein fraction from wild-type mice and from mice with the three mutant genotypes fed a normal chow diet and the 1.25% cholesterol/atherogenic diet. On a normal diet, the LDLR(-/-) mice showed a marked increase in the plasma concentration of apoB-100 and a much smaller relative increase in the concentration of apoB-48 (lane 2). The level of apoE was also increased moderately. In contrast, the apoE(-/-) mice showed a selective increase in apoB-48 without an increase in apoB-100 (lane 3). As expected, apoE was absent. In the apoE(-/-); LDLR(-/-) double homozygotes, the levels of apoB-100 and apoB-48 were both elevated (lane 4). When the LDLR(-/-) mice were fed the 1.25% cholesterol/atherogenic diet, the amount of apoB-48 increased markedly, and there was a dramatic increase in the amount of apoE (compare lanes 6 and 2). There was only a slight increase in apoB-100. When the apoE(-/-) animals were fed the cholesterol/atherogenic diet, the major effect was a marked increase in an apolipoprotein whose molecular weight corresponded to that of apoA-IV (compare lanes 7 and 3). Similar results were observed in the apoE(-/-); LDLR(-/-) double homozygotes (compare lanes 8 and 4). The identity of the protein designated apoA-IV was confirmed by N-terminal peptide sequencing of the protein eluted from the SDS/polyacrylamide gel.

Table 1. Cholesterol content of plasma lipoprotein fractions from mice with different genotypes fed different diets

<table>
<thead>
<tr>
<th>Lipoprotein fraction</th>
<th>Normal chow</th>
<th>1.25% cholesterol</th>
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</thead>
<tbody>
<tr>
<td>LDLR ApoE</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>LDLR +/-</td>
<td>-/-</td>
<td>+/-</td>
</tr>
<tr>
<td>LDLR +/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>LDLR +/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Chylo/VLDL</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>IDL/LDL</td>
<td>17</td>
<td>108</td>
</tr>
<tr>
<td>HDL</td>
<td>68</td>
<td>105</td>
</tr>
</tbody>
</table>

Lipoprotein fractions isolated by FPLC in Fig. 2B were used for measurement of total cholesterol content. The following FPLC fractions were pooled for analysis: chylomicron remnants (Chylo)/VLDL, fractions 4–8; IDL/LDL, fractions 9–18; high density lipoprotein (HDL), fractions 19–27. Data are expressed as mg of cholesterol per dl of original plasma sample (before ultracentrifugation).
The current data are consistent with the notion that apoE*4434 genotypes (Table 4-fold) have been similar in the two mouse mutants. Of chylomicron remnants, the elevation in glycans/LRP, and hence there is no substantial buildup of lipoproteins of apoE(-/-) mice fed a normal chow diet was by the observation that the amount of apoB-48 in the plasma chylomicron remnants. On the other hand, when apoE is this discussion we will refer to the second receptor as LRP, which probably acts in concert with hepatic proteoglycans.

The two-receptor model of remnant clearance is supported by the observation that the amount of apoB-48 in the plasma lipoproteins of apoE(−/−) mice fed a normal chow diet was severalfold higher than that in the LDLR(−/−) mice (Fig. 3). If the LDLR were the only molecule involved in the removal of chylomicron remnants, the elevation in apoB-48 should have been similar in the two mouse mutants.

A more likely possibility is that the apoE on the chylomicron remnants and VLDL of cholesterol-fed mice is insufficient for clearance through the proteoglycan/LRP complex. Previous data demonstrate that apoE on β-VLDL particles isolated from cholesterol-fed rabbits is insufficient for binding to LRP. To demonstrate this binding, it is necessary to add additional amounts of exogenous apoE (18). It is possible that the increased surface area of large cholesterol-rich particles dictates a requirement for increased apoE in order to achieve a high enough local concentration on the particle’s surface to permit high-affinity binding to LRP. Although apoE increases in the cholesterol-fed LDLR(−/−) mice, the increase may be insufficient to maintain high-affinity binding to LRP. Previous studies have shown that the C apolipoproteins decrease the binding effectiveness of apoE (20, 21). We have not yet studied the C apolipoproteins in the mutant mice.

A new finding in this study is the marked increase in lipoprotein-bound apoA-IV in the circulation of the apoE-deficient mice that were fed the high cholesterol/atherogenic diet. The increase was the same whether or not the LDLR was present. ApoA-IV, an apolipoprotein of unknown function, is produced in the intestine and secreted on chylomicrons. The total plasma concentration of apoA-IV is increased by fat feeding in rats (22) and humans (23). The increase in apoA-IV concentration elicited by the 1.25% cholesterol/atherogenic diet was much greater in the apoE-deficient mice than in the wild-type mice. The functional significance of this observation is obscure, but it may be related to the observation that apoA-IV activates lecithin cholesterol acyltransferase (24). ApoA-IV may increase in order to enhance cholesterol esterification in the plasma of the cholesterol-overloaded mice. A substantial amount of apoA-IV is normally present in the nonlipoprotein fraction of plasma (23). We do not yet know whether the total amount of apoA-IV increases in the plasma of the cholesterol-fed apoE(−/−) mice or whether there is a transfer from the nonlipoprotein fraction to particles that are devoid of apoE.

Table 2. Liver cholesterol content in mice with different genotypes fed different diets

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Liver cholesterol content, mg per g of tissue</th>
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<tbody>
<tr>
<td>LDLR</td>
<td>ApoE</td>
</tr>
<tr>
<td>+/-</td>
<td>Normal chow</td>
</tr>
<tr>
<td>-/-</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>+/-</td>
<td>5.1 ± 0.6</td>
</tr>
<tr>
<td>-/-</td>
<td>4.6 ± 0.9</td>
</tr>
<tr>
<td>+/-</td>
<td>6.1 ± 0.4</td>
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Total cholesterol content in liver was measured in female mice with the indicated genotype (*n* = 3) fed the indicated diet for 15 days. Mean age of mice was 11 weeks at the beginning of the study. Values are means ± SE.

The 1.25% cholesterol/atherogenic diet caused a massive increase in the cholesterol content of the chylomicron/VLDL and IDL/LDL fractions in animals of all three genotypes. The relative increase was highest in the LDLR(−/−) animals (=100-fold increase in chylomicron/VLDL cholesterol). This is because the LDLR(−/−) animals on a chow diet had much lower levels of chylomicron/VLDL cholesterol as compared with the apoE(−/−) mice and the apoE(−/−)/; LDLR(−/−) double homozygotes. The 1.25% cholesterol/atherogenic diet also caused a marked increase in the apoB-48 level of the LDLR(−/−) mice (Fig. 3, lane 6).

Why should the high cholesterol/atherogenic diet cause such a profound elevation in the content of cholesterol and apoB-48 in the chylomicron/VLDL fraction in LDLR(−/−) animals? These animals have abundant apoE. Indeed, the apoE concentration increases markedly on the high cholesterol diet. Why doesn’t apoE prevent the hypercholesterolemia by binding to LRP? It seems unlikely that LRP becomes saturated as a result of the overproduction of chylomicron remnants and VLDL on the high cholesterol/atherogenic diet. Abundant amounts of LRP are normally present in the liver. At saturation, LRP should be carrying large amounts of chylomicron remnant particles into the liver, and one would expect that the levels of apoB-48 and cholesterol in the cholesterol-fed LDLR(−/−) mice should be substantially lower than in the cholesterol-fed apoE(−/−); LDLR(−/−) mice in which LRP cannot operate. This was not the case.

Fig. 3. SDS/PAGE of apolipoproteins from total lipoprotein fractions (d < 1.215 g/ml) from mice of different genotypes fed different diets. The total fra.
tein particles will undoubtedly provide further insights into lipoprotein physiology.

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