Hyperlipidemia of ApoE2(Arg158-Cys) and ApoE3-Leiden Transgenic Mice Is Modulated Predominantly by LDL Receptor Expression

Ko Willems van Dijk, Bart J.M. van Vlijmen, Menno P.J. de Winther, Belinda van ’t Hof, Andre van der Zee, Hans van der Boom, Louis M. Havekes, Marten H. Hofker

Abstract—To investigate the relative roles of the LDL receptor– and non–LDL receptor–mediated pathways in the clearance of apolipoprotein E (apoE) variants in vivo, we have generated apoE2(Arg158-Cys) (apoE2) and apoE3-Leiden transgenic mice deficient for the endogenous mouse Apoe and Ldlr receptor genes (Apoe−/−Ldlr−/− mice). Unexpectedly, on the Apoe−/−Ldlr−/− background, expression of neither apoE2 nor apoE3-Leiden results in a decrease of the hyperlipidemia. In contrast, serum cholesterol levels are increased by the introduction of apoE2 and apoE3-Leiden in Apoe−/−Ldlr−/− mice (to 39.1±7.1 and 37.6±7.6 mmol/L, respectively, from 25.9±6.5 mmol/L). In addition, in these transgenic mice, the serum triglyceride levels are substantially increased (to 9.6±7.0 and 5.8±2.8 mmol/L, respectively, from 0.7±0.5 mmol/L), which is associated with a decreased efficiency of in vitro LPL-mediated lipolysis of circulating VLDL. The VLDL-triglyceride secretion rate is not affected by the expression of apoE2 or apoE3-Leiden on the Apoe−/−Ldlr−/− background. These results indicate that in the absence of the LDL receptor, clearance of triglyceride-rich apoE2 and apoE3-Leiden–containing lipoproteins via alternative hepatic receptors, such as the LDL receptor–related protein (LRP) is inefficient. Although apoE2 and apoE3-Leiden are disturbed in binding to the LDL receptor in vitro, expression of 1 or 2 mouse Ldlr alleles in an apoE2.Apoe−/− or apoE3-Leiden.Apoe−/− background results in a gene dose–dependent decrease of the hyperlipidemia. Furthermore, overexpression of the LDL receptor via adenovirus-mediated gene transfer rescues the hyperlipidemia associated with apoE2 and apoE3-Leiden expression. These data indicate that in apoE2 and apoE3-Leiden transgenic mice, the LDL receptor constitutes the predominant route for clearance of VLDL remnants, carrying even poorly binding apoE variants, and that this pathway is functional despite an apoE-mediated disturbance in VLDL triglyceride lipolysis. (Arterioscler Thromb Vasc Biol. 1999;19:2945-2951.)

Key Words: apolipoprotein E • LDL receptor • LDL receptor–related protein • hypertriglyceridemia • VLDL triglyceride lipolysis

A polipoprotein (apo) E plays a key role in the lipoprotein metabolism by functioning as a ligand for receptor-mediated uptake of chylomicron and VLDL remnants by the liver (for review, see Reference 1). Mutations in apoE are associated with familial dysbeta1ipoproteinemia (FD), which is characterized by elevated levels of chylomicron and VLDL remnants in the serum and premature atherosclerosis (for reviews, see References 2 and 3). Several variant forms of apoE leading to FD have been described, such as the common apoE2(Arg158-Cys) (apoE2) variant, which is inherited as a recessive trait, and the rare apoE3-Leiden variant, which is inherited as a dominant trait.¹

The penetrance and severity of FD associated with the apoE2 and apoE3-Leiden variants is variable,¹,³ most likely because of the genetic and environmental heterogeneity of the human population. To investigate the role of these apoE variants in FD in a more homogeneous background, we have previously generated transgenic mice expressing the human apoE2 and apoE3-Leiden variants in the absence of endogenous mouse apoE.⁵ Both mouse lines display hyperlipidemia, but to a different extent. ApoE2.Apoe−/− mice are much more affected than apoE3-Leiden.Apoe−/− mice. These differences could be partly explained by in vitro binding studies that revealed a severe binding defect of apoE2 and a moderate binding defect of apoE3-Leiden to the LDL receptor.⁵

In addition to the LDL receptor, the LDL receptor–related protein (LRP) is thought to function as a backup receptor mediating clearance of apoE-containing lipoproteins.⁶–⁸ This has been demonstrated by the accumulation of chylomicron...
and VLDL remnants in the serum of LDL receptor–deficient (Ldlr–/–) mice injected with an adenovirus carrying the receptor-associated protein (ad-RAP), a potent inhibitor of LRP-ligand interaction.10 Similar observations have been made after liver-specific inactivation of the LRP gene in Ldlr–/– mice by a genetic strategy.11 Inhibition of the LRP by ad-RAP injection in apoE2.Apoε–/– and apoE3-Leiden.

 apoE–/– mice resulted in a dramatic increase of the hyperlipidemia.2 These results were interpreted to indicate that both apoE2- and apoE3-Leiden–containing lipoproteins can be cleared via the LRP.

To further investigate the clearance of apoE2- and apoE3-Leiden–containing lipoproteins via non–LDL receptor–mediated pathways, we generated transgenic mice expressing both apoE variants on an Apoε–/– and LDL receptor–deficient background (apoE2.Apoε–/–.Ldlr–/– and apoE3-Leiden.Apoε–/–.Ldlr–/– mice). These mice were compared with Apoε–/–.Ldlr–/– mice and with apoE2.Apoε–/– and apoE3-Leiden.Apoε–/– mice with varying levels of LDL receptor expression. Our data indicate that despite decreased in vitro binding to the LDL receptor, apoE2 and apoE3-Leiden lipoproteins are cleared predominantly via the LDL receptor in vivo. In addition, compared with LRP-mediated clearance, LDL receptor–mediated clearance seems less sensitive to an apoE2- and apoE3-Leiden–mediated defect in VLDL triglyceride lipolysis.

Methods

Generation and Analysis of Transgenic Mice

Transgenic mice expressing human apoE2(Arg158-Cys) and apoE3-Leiden in the absence of endogenous apoE have been described previously13 (apoE2.Apoε–/– and apoE3-Leiden.Apoε–/– mice). LDL receptor–deficient (Ldlr–/–) mice12 were purchased from the Jackson Laboratory (Bar Harbor, Me). ApoE2.Apoε–/– and apoE3-Leiden.Apoε–/– mice were crossed with Ldlr–/– mice to obtain transgenic mice that lack both the apoE and LDL receptor gene (apoE2.Apoε–/–.Ldlr–/– and apoE3-Leiden.Apoε–/–.Ldlr–/– mice). The resulting breeding offspring were analyzed for the endogenous Apoε–/– and Ldlr–/– genotype through tail-tip DNA analysis, as described earlier.12,13 The presence of human apoE in serum was determined by sandwich ELISA as described previously.14 For experiments, mice 10 to 20 weeks of age were included. Mice were housed under standard conditions in conventional cages and given free access to food (ie, a standard chow diet) and water. All experiments were performed under protocols approved by the Committee on Animal Experimentation of the Leiden University Medical Center.

Lipid, Lipoprotein, and ApoE Measurements

Mice were fasted from 9 AM to 1 PM, and ~150 µL of blood was obtained from each individual mouse through tail-bleeding. Total serum cholesterol and triglyceride levels (without measurement of free glycerol) were measured enzymatically with commercially available kits: Boehringer Mannheim 236691 and Sigma Chemical Co 337-B, and Wako Chemicals GmbH 990-54009, respectively). VLDL protein was determined by the method of Lowry.15 Human apoE levels were measured by sandwich ELISA as described previously.14

Adenovirus Transfections

The recombinant adenoviral vectors expressing the human LDL receptor (Ad-LDLR) and the bacterial β-galactosidase (Ad-LacZ) under control of the cytomegalovirus promotor were kindly provided by Dr T. Willnow (University of Texas Southwestern Medical Center, Dallas) and Dr J. Herz (Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany).10 The recombinant adenovirus was propagated and titrated on the Ad5 E1–transformed human embryonic kidney cell line 911 as described.16 For storage, the virus was supplemented with mouse serum albumin (0.2%) and glycerol (10%), and aliquots were flash-frozen in liquid N2 and stored at −80°C. Routine virus titers of the stocks varied from 1×1010 to 5×1010 pfu/mL.

For in vivo adenovirus transfection, on day 0, 2×1010 pfu in a total volume of 200 µL (diluted with PBS) were injected into the tail vein. Blood samples were drawn from the tail vein of fasted mice at 5 days after virus injection.

In Vivo Hepatic VLDL-Triglyceride Production

After a 4-hour fasting period, mice were injected intravenously with Triton WR1339 (500 mg/kg body wt)17 with 15% (wt/vol) Triton solution in 0.9% NaCl. At 1, 20, 40, and 60 minutes after injection, blood samples were drawn and analyzed for triglycerides as described above. The increase in serum triglycerides was normalized to the 1-minute point. Production of hepatic triglyceride was calculated from the slope of the curve and expressed as µmol·h−1·kg body wt−1.

Assay of Lipolysis With Lipoprotein Lipase in Solution

The d<1.006 (g/mL) lipoproteins (VLDL) were isolated by density-gradient ultracentrifugation. Lipolysis assays were performed at 37°C in a 0.1 mol/L Tris(hydroxymethyl)-aminomethane (Tris) buffer, pH 8.5, with bovine lipoprotein lipase (LPL: 0.2 U, Sigma) in the presence of 2% (wt/vol) bovine albumin (essentially free of free fatty acids [FFAs]). The reaction was stopped by the addition of 50 mmol/L KH2PO4, 0.1% Triton X-100, pH 6.9, and placed on ice. To obtain a time 0 control, the reaction was prevented by addition of Triton before the addition of LPL, and samples were placed on ice. FFAs were measured enzymatically with a NeFa-C kit (Wako Chemicals GmbH). The rate of FFA release by 0.2 U LPL was linear for 5 minutes as used in this assay. The assay was performed at a VLDL-triglyceride concentration of 0.14 mmol/L with duplication of FFA determination.

Results

Serum Lipid, ApoE, and Lipoprotein Levels in ApoE Transgenic Apoε–/–.Ldlr–/– Mice

To determine the relative roles of the LDL receptor–versus the non–LDL receptor–mediated pathways in the clearance of apoE variants, apoE2 and apoE3-Leiden transgenic mice were crossbred onto an Apoε–/–.Ldlr–/– background. ApoE2 and apoE3-Leiden mice express their transgenes predominantly in the liver. Both apoE2.Apoε–/–.Ldlr–/– and apoE3-Leiden.Apoε–/–.Ldlr–/– mice are even more severely hyperlipidemic than nontransgenic Apoε–/–.Ldlr–/– mice (Table 1). Compared with Apoε–/–.Ldlr–/– mice, the presence of apoE2 and apoE3-Leiden leads to an aggravation of the hypercholesterolemia and the appearance of severe hypertriglyceridemia. Both apoE2.Apoε–/–.Ldlr–/– and
apoE3-Leiden. Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> mice have human apoE levels of >20 mg/dL. As determined by size-separation chromatography with an FPLC apparatus, the increase in serum cholesterol and triglycerides in the apoE2, Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> and apoE3-Leiden. Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> mice occurred predominantly in the fractions belonging to the VLDL/IDL size range (Figure 1).

**LDL Receptor Expression in ApoE Transgenic Mice**

We have previously reported the generation and analysis of apoE2 and apoE3-Leiden transgenic mice on an Apoe<sup>−/−</sup>.Ldlr<sup>+/+</sup> background. These mice have a significantly less pronounced hyperlipidemia than apoE2 and apoE3-Leiden transgenic mice on an Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> background (Table 1). Interestingly, apoE2. Apoe<sup>−/−</sup> and apoE3-Leiden. Apoe<sup>−/−</sup> mice heterozygous for LDL receptor deficiency (Ldlr<sup>−/+</sup>) have serum lipid levels that are intermediate between complete absence or presence of the LDL receptor (Table 1). Apparently, the murine LDL receptor is capable of mediating clearance of VLDL carrying apoE variants that bind poorly in in vitro assays.

To further demonstrate the capacity of the LDL receptor to mediate clearance of apoE2 and apoE3-Leiden, the human LDL receptor was overexpressed in apoE2 and apoE3-Leiden transgenic mice by adenovirus-mediated gene transfer. Injection of 2×10<sup>9</sup> pfu of Ad-LDLR in Apoe<sup>−/−</sup> mice does not affect serum cholesterol levels, whereas a similar dose of Ad-LDLR in Ldlr<sup>−/−</sup> mice reduces the serum cholesterol levels to that of a wild-type mouse. In apoE2. Apoe<sup>−/−</sup> and apoE3-Leiden. Apoe<sup>−/−</sup> mice, injection of 2×10<sup>9</sup> pfu of Ad-LacZ. The reduction in serum triglyceride and apoE levels shows a similar trend (Table 2). As illustrated by size separation chromatography, the reduction in serum lipids in apoE2. Apoe<sup>−/−</sup> and apoE3-Leiden. Apoe<sup>−/−</sup> mice occurs across the whole spectrum of VLDL-, IDL-, and LDL-sized particles (Figure 2).

**Characterization of the d<1.006 Lipoproteins From ApoE Transgenic Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> Mice**

The composition of the d<1.006 lipoproteins (VLDL) was determined to further characterize the hyperlipidemia associated with apoE2 and apoE3-Leiden expression in the absence of the LDL receptor. Sets of pooled serum from Ldlr<sup>−/−</sup>-deficient mice that express endogenous mouse Apoe, no Apoe, apoE2, and apoE3-Leiden were fractionated by density ultracentrifugation. The lipid and apoE content of the various VLDL particles is shown in Table 3. The total cholesterol and triglyceride levels in the apoE transgenic VLDL are significantly increased compared with non--apoE transgenic VLDL. Concomitantly with the increased apolar lipid content (free cholesterol and triglycerides) of the apoE2 and apoE3-Leiden VLDL and thus an expected size increase, the surface resident phospholipid levels are elevated. A significant fraction (≈20%) of the total VLDL protein consists of apoE.

**Analysis of the Disturbance in Triglyceride Metabolism in ApoE Transgenic Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> Mice**

The concomitant increase in serum triglyceride and apoE levels in the apoE2 and apoE3-Leiden transgenic mice is indicative of an apoE-mediated defect in the triglyceride metabolism (Table 1). This was further supported by a strong

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**TABLE 1. Serum Lipid and ApoE Levels in ApoE Transgenic Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> Mice**

<table>
<thead>
<tr>
<th>Transgene Deficiency</th>
<th>n</th>
<th>TC, mmol/L</th>
<th>TG, mmol/L</th>
<th>ApoE, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>... Apoe&lt;sup&gt;−/−&lt;/sup&gt;.Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>30</td>
<td>25.9±6.5</td>
<td>0.7±0.5</td>
<td>...</td>
</tr>
<tr>
<td>ApoE2 Apoe&lt;sup&gt;−/−&lt;/sup&gt;.Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>5</td>
<td>39.1±7.1</td>
<td>9.6±7.0</td>
<td>25.9±5.7</td>
</tr>
<tr>
<td>ApoE2 Apoe&lt;sup&gt;−/−&lt;/sup&gt;.Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>3</td>
<td>26.5±4.3</td>
<td>4.7±0.9</td>
<td>ND</td>
</tr>
<tr>
<td>ApoE3-Leiden Apoe&lt;sup&gt;−/−&lt;/sup&gt;.Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>15</td>
<td>16.5±2.9</td>
<td>2.4±0.8</td>
<td>9.2±0.8</td>
</tr>
<tr>
<td>ApoE3-Leiden Apoe&lt;sup&gt;−/−&lt;/sup&gt;.Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>12</td>
<td>37.6±7.6</td>
<td>5.8±2.8</td>
<td>21.3±7.3</td>
</tr>
<tr>
<td>ApoE3-Leiden Apoe&lt;sup&gt;−/−&lt;/sup&gt;.Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>7</td>
<td>13.4±4.0</td>
<td>1.1±0.8</td>
<td>5.2±0.6</td>
</tr>
<tr>
<td>ApoE3-Leiden Apoe&lt;sup&gt;−/−&lt;/sup&gt;.Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>15</td>
<td>3.6±1.5</td>
<td>0.3±0.2</td>
<td>0.5±0.1</td>
</tr>
</tbody>
</table>

Values shown are from female mice 10 to 20 weeks old fed a standard chow diet. Blood was drawn from the tail vein after a 4-hour fast. Serum cholesterol (TC), triglyceride (TG), and apoE levels were determined. ND indicates not determined.

*Data for Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> and Apoe3-Leiden. Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> mice from Reference 5.

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**Figure 1.** Distribution of cholesterol and triglycerides among lipoprotein fractions. Lipoprotein fractions were separated by FPLC permeation chromatography with two 25-ml Superose 6B columns in series, and fractions were analyzed for cholesterol (solid line) and triglycerides (dashed line). Lipoprotein profiles of Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> mice (A), apoE2. Ldlr<sup>−/−</sup>/Apoe<sup>−/−</sup> mice (B), and apoE3-Leiden. Ldlr<sup>−/−</sup>/ Apoe<sup>−/−</sup> mice (C) are shown. Each run is performed with a fasted serum pool of 5 to 8 female mice. The horizontal lines indicate the fractions in which the various lipoproteins (VLDL, IDL, LDL, and HDL) are present.
Table 2. Effect of Systemic ad-LDLR Administration to ApoE2/ApoE−/− and ApoE3-Leiden/ApoE−/− Mice on Serum Lipid and Human ApoE Levels

<table>
<thead>
<tr>
<th>Transgene Deficiency</th>
<th>Mouse</th>
<th>Preinjection</th>
<th>Ad-LacZ</th>
<th>Ad-LDLR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TC, mmol/L</td>
<td>TG, mmol/L</td>
<td>ApoE, mg/dL</td>
</tr>
<tr>
<td>Ldlr−/−</td>
<td></td>
<td>21.3±4.1</td>
<td>4.2±1.2</td>
<td>17.0±4.9</td>
</tr>
<tr>
<td>Ldlr−/−</td>
<td></td>
<td>9.5±2.2</td>
<td>0.7±0.6</td>
<td>3.4±1.0</td>
</tr>
<tr>
<td>ApoE2</td>
<td></td>
<td>14.9±6.4</td>
<td>4.9±1.3</td>
<td>3.2±0.9</td>
</tr>
<tr>
<td>ApoE3-Leiden†</td>
<td></td>
<td>14.9±6.4</td>
<td>4.9±1.3</td>
<td>3.2±0.9</td>
</tr>
</tbody>
</table>

Female mice 10 to 15 weeks old were given 2×10⁸ pfu Ad-LDLR or Ad-LacZ. Values are shown for fasted blood samples drawn from the tail vein 2 days before (preinjection) and 5 days after adenovirus injection. TC indicates total cholesterol; TG, triglycerides; and apoE, human apoE concentration.

Positive correlation between the serum apoE and triglyceride levels and not the serum apoE and cholesterol levels in the individual apoE2/ApoE−/− Ldlr−/− and apoE3-Leiden/ApoE−/− Ldlr−/− mice (data not shown). The pronounced hypertriglyceridemia in the apoE transgenic ApoE−/− Ldlr−/− mice compared with the nontransgenic ApoE−/− Ldlr−/− mice could be explained by an apoE-induced increase in VLDL-triglyceride production and/or a decrease in the efficiency of triglyceride lipolysis.

To determine whether apoE expression affects VLDL-triglyceride production, ApoE−/− Ldlr−/−, apoE2/ApoE−/− Ldlr−/−, and apoE3-Leiden/ApoE−/− Ldlr−/− mice were injected with Triton WR1339, and the serum triglyceride increase was determined. As evident from Table 4, differences in the VLDL-triglyceride secretion rate cannot explain the observed hypertriglyceridemia in the apoE transgenic mice on the ApoE−/− Ldlr−/− background.

To investigate whether increased amounts of apoE2 and apoE3-Leiden have an effect on the efficiency of VLDL lipolysis, VLDL from Ldlr−/− mice carrying normal amounts of endogenous mouse ApoE and VLDL from apoE2/ApoE−/− Ldlr−/− and apoE3-Leiden/ApoE−/− Ldlr−/− mice carrying excess human apoE variants (Table 3) were subjected to lipolysis by bovine LPL in solution. Both apoE2 and apoE3-Leiden VLDL is lipolysed at <20% of the efficiency of VLDL containing endogenous mouse apoE (Table 4).

Discussion

In this article, we demonstrate that compared with ApoE−/− Ldlr−/− mice, expression of apoE2 and apoE3-Leiden on an ApoE−/− Ldlr−/− background does not result in a reduction of the hyperlipidemia. On the contrary, both serum cholesterol and triglyceride levels are dramatically increased in apoE2/ApoE−/− Ldlr−/− mice and apoE3-Leiden/ApoE−/− Ldlr−/− mice (Table 1). The hypertriglyceridemia can be explained by a poor efficiency of lipolysis of the apoE-rich VLDL particles (Table 4). Because expression of the apoE

Figure 2. Distribution of serum cholesterol and triglycerides among lipoprotein fractions after adenovirus-mediated gene transfer. Lipoprotein fractions were separated by FPLC permeation chromatography with two 25-ml Superose 6B columns in series, and fractions were analyzed for cholesterol (solid lines) and triglycerides (dashed lines). Lipoprotein profiles of ApoE−/− mice before injection (A) and 5 days after Ad-LDLR injection (B); Ldlr−/− mice before injection (C) and 5 days after Ad-LDLR injection (D); apoE2/ApoE−/− mice 5 days after Ad-LacZ injection (E) and 5 days after Ad-LDLR injection (F); and apoE3-Leiden/ApoE−/− mice 5 days after Ad-LacZ injection (G) and 5 days after Ad-LDLR injection (H) are shown. The apoE3-Leiden/ApoE−/− were fed a western-type diet with 0.5% cholesterol for 4 weeks. Each run was performed with a fasted serum pool of 4 to 8 mice (groups from Table 2). The horizontal lines indicate the fractions in which the various lipoproteins (VLDL, IDL, LDL, and HDL) are present.
variants on the Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> background does not affect the hepatic VLDL-triglyceride secretion rate (Table 4), we conclude that the increase in hypercholesterolemia is due to dysfunctional VLDL clearance via a backup receptor pathway such as the LRP. Despite decreased in vitro binding of apoE2 and apoE3-Leiden VLDL to the LDL receptor<sup>5</sup> and impaired triglyceride lipolysis, increasing the expression of the LDL receptor in both apoE2.Apo<sup>−/−</sup>.Ldlr<sup>−/−</sup> and apoE3-Leiden.Apo<sup>−/−</sup>.Ldlr<sup>−/−</sup> mice by breeding or by Ad-LDLR injection results in a gene dose–dependent decrease of the hyperlipidemia (Tables 1 and 2).

Increased LDL receptor expression in apoE3-Leiden mice results in a much more efficient reduction of serum cholesterol level than in apoE2 mice (Tables 1 and 2). Only excess LDL receptor expression after adenovirus-mediated gene transfer results in a nearly complete rescue of the hyperlipidemia in apoE2.Apo<sup>−/−</sup>.Ldlr<sup>−/−</sup> mice. This is in agreement with the poor in vitro LDL receptor binding capacity of apoE2 compared with apoE3-Leiden<sup>5</sup> and, moreover, sustains our conclusion that the LDL receptor is the predominant pathway for lipoprotein clearance in apoE2 and apoE3-Leiden transgenic mice.

In vivo, LRP-mediated clearance is thought to occur after enrichment of the remnant lipoproteins with apoE in the space of Disse, the so-called secretion-recapture process. Our data clearly indicate that LRP-mediated clearance of lipoproteins from the circulation of apoE2.Apo<sup>−/−</sup>.Ldlr<sup>−/−</sup> and apoE3-Leiden.Apo<sup>−/−</sup>.Ldlr<sup>−/−</sup> mice is disturbed, despite high levels of apoE on the VLDL particles (Table 3). Explanations for disturbed LRP-mediated clearance include defective lipoprotein binding caused by the apoE variants and/or the lipid composition of the particles. We and others have recently demonstrated in mice that excess apoE3, the most common apoE variant in humans, on VLDL particles also leads to inhibition of VLDL-triglyceride lipolysis and a disturbed clearance via the LRP.<sup>22,23</sup> The present observations demonstrate that the triglyceride-rich VLDL from apoE2.Apo<sup>−/−</sup>.Ldlr<sup>−/−</sup> and apoE3-Leiden.Apo<sup>−/−</sup>.Ldlr<sup>−/−</sup> mice is poorly cleared via the LRP. We conclude from the combined data that the high triglyceride content of the VLDL particles is one of the factors contributing to defective LRP-mediated clearance in the apoE2.Apo<sup>−/−</sup>.Ldlr<sup>−/−</sup> and apoE3-Leiden.Apo<sup>−/−</sup>.Ldlr<sup>−/−</sup> mice.

Disturbance of VLDL-triglyceride lipolysis by variant forms of human apoE has been reported previously.<sup>24–26</sup> However, this negative effect on triglyceride lipolysis may be partly apoE isotype independent, because wild-type apoE3 can also decrease the efficiency of triglyceride lipolysis when present at high levels on the VLDL<sup>23,27–30</sup>. On the basis of our experiments, we cannot distinguish quantitative effects from qualitative effects of apoE2 and apoE3-Leiden on the efficiency of triglyceride lipolysis. It has recently been described that increased levels of mouse apoE in the serum of Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> mice do not result in hypertriglyceridemia.<sup>31</sup> This indicates that increased human and mouse apoE levels may have distinct effects on the efficiency of triglyceride lipolysis.

The role of the LRP in lipoprotein clearance has recently been investigated by liver-specific ablation of LRP expression.<sup>11</sup> On an Ldlr<sup>−/−</sup> background, absence of the LRP from the liver results in the accumulation of lipoproteins in the VLDL and IDL/IDL size range. On a wild-type Ldlr<sup>+/+</sup> background, absence of the LRP from the liver does not result in the accumulation of lipoproteins in the circulation but does result in a compensatory upregulation of the endogenous LDL receptor gene and protein. These data provide direct evidence for a role of the LRP in lipoprotein clearance but also emphasize the relative importance of the LDL receptor for lipoprotein clearance, which is in line with our present conclusions.

The conditional LRP knockout experiments have also provided novel insight into the effects of adenovirus-mediated overexpression of RAP, a potent inhibitor of the LRP.<sup>9</sup> Injection of Ad-RAP in Ldlr<sup>−/−</sup> mice results in a much more dramatic accumulation of VLDL-size lipoproteins<sup>10</sup> than with Ldlr<sup>−/−</sup> mice lacking the LRP exclusively from the liver.<sup>11</sup> Apparently, overexpression of RAP not only inhibits lipoprotein interaction with the LRP but also inter-

### TABLE 3. Composition of d<1.006 Lipoproteins From ApoE Transgenic Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> Mice

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Deficiency</th>
<th>TC</th>
<th>CE</th>
<th>FC, μmol/mg Protein</th>
<th>TG</th>
<th>PL</th>
<th>ApoE, mg/mg Protein</th>
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</thead>
<tbody>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.6±0.06</td>
<td>0.3±0.0</td>
<td>0.30±0.06</td>
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<td>...</td>
</tr>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;.Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>9.8</td>
<td>7.3</td>
<td>2.5</td>
<td>0.6</td>
<td>1.7</td>
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<td>14.9±0.1</td>
<td>10.4±0.1</td>
<td>4.5±0.2</td>
<td>2.5±0.6</td>
<td>3.4±0.2</td>
<td>0.2±0.02</td>
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<td>Apoe3-Leiden</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;.Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>17.4±1.7</td>
<td>11.8±0.9</td>
<td>5.6±0.9</td>
<td>4.8±1.4</td>
<td>4.0±0.7</td>
<td>0.2±0.1</td>
</tr>
</tbody>
</table>

Chow-fed male and female mice were fasted and bled via orbital puncture. d<1.006 lipoproteins were isolated from sets of serum pools of 3 mice by density-gradient ultracentrifugation. The d<1.006 fractions were analyzed for total (TC), esterified (CE), and free (FC) cholesterol, triglycerides (TG), phospholipids (PL), and human apoE and are expressed per mg VLDL protein.

### TABLE 4. In Vivo Hepatic Triglyceride Production Rate and In Vitro Triglyceride Lipolysis Efficiency of VLDL Derived From ApoE Transgenic Apoe<sup>−/−</sup>.Ldlr<sup>−/−</sup> Mice

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Deficiency</th>
<th>Hepatic TG Production, µmol · kg&lt;sup&gt;−1&lt;/sup&gt; · h&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>FFA Release, mmol</th>
<th>FFA/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>ND</td>
<td>9.7±1.4</td>
<td></td>
</tr>
<tr>
<td>Apoe2</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;.Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>75.4±16.8</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Apoe3-Leiden</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;.Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>77.8±12.9</td>
<td>1.1±1.2</td>
<td></td>
</tr>
</tbody>
</table>

Female mice (n=3–6 per group, 19 weeks old) were fasted, anesthetized, and injected with Triton WR 1339 IV. At 1, 20, 40, and 60 minutes after injection, blood samples were drawn and serum triglycerides (TG) were determined. The d<1.006 lipoproteins as characterized in Table 3 were used for an in vitro lipolysis assay. FFA release by bovine LPL in solution was determined at a triglyceride substrate concentration of 0.14 mmol/L. ND indicates not determined.
fere with additional steps in the metabolism of VLDL-size lipoproteins. We have previously demonstrated that adenovirus-mediated overexpression of RAP in apoE2. ApoE*2/−/− and apoE3-Leiden.ApoE*2/−/− mice results in a dramatically increased hyperlipidemia.5 Given the recent information on the effects of RAP on lipoprotein metabolism, we cannot exclude the possibility that part of this observed phenotype is due to additional disturbances of lipoprotein metabolism.

Huang et al12 generated apoE2 transgenic mice expressing various plasma levels of apoE2 (3 to 60 mg/dL) in the presence of endogenous mouse apoE (apoE2.ApoE*/+/- mice). In moderately expressing apoE2.ApoE*/+/- mice (10 to 30 mg/dL human apoE2), absence of one or both LDL receptor alleles resulted in a LDL receptor gene dose–dependent hyperlipidemia,33 similar to what we observe. Surprisingly, in low-expressing apoE2.ApoE*/+/- mice (2 to 10 mg/dL human apoE2), absence of the LDL receptor was found to result in hypolipidemia.26 These authors concluded from their data that these phenotypes can be explained by the combined effects of both defective LDL receptor–mediated clearance and an apoE2-mediated disturbance of triglyceride lipolysis, which is fully in line with our present conclusions.

The apoE2.ApoE*/+/- mice used to generate the apoE2.ApoE*/−/−.Ldlr*−/− mice in the present study express levels of apoE similar to those of the low-expressing mice used by Huang et al.26 However, in contrast to the mice used by Huang et al, our low-expressing apoE2.ApoE*/−/−.Ldlr*−/− mice are severely hyperlipidemic compared with both Ldlr*−/− mice and even ApoE*/−/−.Ldlr*−/− mice (Table 1). The apparent discrepancy in the plasma cholesterol and triglyceride levels of apoE2 mice on the ApoE2/Ldlr*−/− background (this study) versus the ApoE*/−/−.Ldlr*−/− background (this study) may be due to the apparent beneficial effects of mouse apoE on clearance and lipolysis.

It has been reported that humans heterozygous for LDL receptor deficiency have increased hyperlipidemia in the presence of 1 or 2 apoE2 alleles.34,35 These observations in the human agree with the present data on the relative importance of the LDL receptor for the clearance of lipoproteins containing even poorly binding apoE variants in the mouse. Thus, individual variation in the level of LDL receptor expression could play an important role in the expression of hyperlipidemia even in individuals expressing mutant forms of apoE that bind poorly to the LDL receptor.

Acknowledgments

This research was supported by the Netherlands Heart Foundation (projects M93.001 and 96.178) and the Netherlands Foundation of Scientific Research (project 900-504-092). Financial contribution of the specific RTD Program of the European Commission (BIOMED-2, BMR4-CT96-0898) is gratefully acknowledged. Dr Hofker is an Established Investigator of the Netherlands Heart Foundation. We gratefully acknowledge the technical assistance of V.E.H. Dahlmans.

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doi: 10.1161/01.ATV.19.12.2945

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