Apolipoprotein E2 Reduces the Low Density Lipoprotein Level in Transgenic Mice by Impairing Lipoprotein Lipase-mediated Lipolysis of Triglyceride-rich Lipoproteins*

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Apolipoprotein (apo) E2 is often associated with low levels of low density lipoprotein (LDL) cholesterol and high levels of plasma triglycerides in humans. Mice expressing apoE2 also have low LDL levels. To evaluate the possible role of the LDL receptor in the cholesterol-lowering effect of apoE2, we bred transgenic mice expressing low levels of apoE2 with LDL receptor-null mice (hE2+/0,LDLR−/−). Even in the absence of the LDL receptor, plasma total and LDL cholesterol levels decreased progressively with increasing levels of plasma apoE2. At plasma apoE2 levels >20 mg/dl, LDL cholesterol was ~45% lower than in LDLR−/− mice. Thus, the LDL cholesterol-lowering effect of apoE2 is independent of the LDL receptor. In contrast, plasma triglyceride levels increased (mostly in very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL)) progressively as apoE2 levels increased. At plasma apoE2 levels >20 mg/dl, triglycerides were ~150% higher than in LDLR−/− mice. Furthermore, in apoE-null mice (hE2+/0, mE+/+), apoE2 levels also correlated positively with plasma triglyceride levels, suggesting impaired lipolysis in both hE2+/0,LDLR−/− and hE2+/0, mE+/+ mice. Incubating VLDL or IDL from the hE2+/0,LDLR−/− or the hE2+/0, mE+/+ mice with mouse postheparin plasma inhibited lipoprotein lipease-mediated lipolysis of apoE2-containing VLDL and IDL by ~80 and ~70%, respectively, versus normal VLDL and IDL. This observation was confirmed by studies with triglyceride-rich emulsion particles, apoE2, and purified lipoprotein lipase. Furthermore, apoE2-containing VLDL had much less apoC-II than normal VLDL. Adding apoC-II to the incubation partially corrected the apoE2-impaired lipolysis in apoE2-containing VLDL or IDL and corrected it completely in apoE2-containing emulsion particles. Thus, apoE2 lowers LDL cholesterol by impairing lipoprotein lipase-mediated lipolysis of triglyceride-rich lipoproteins (mostly by displacing or masking apoC-II). Furthermore, the effects of apoE2 on both plasma cholesterol and triglyceride levels are dose dependent and act via different mechanisms. The increase in plasma cholesterol caused by apoE2 is due mostly to impaired clearance, whereas the increase in plasma triglycerides is caused mainly by apoE2-impaired lipolysis of triglyceride-rich lipoproteins.

Apolipoprotein (apo) E polymorphism is one of the common genetic factors responsible for interindividual differences in plasma lipid and lipoprotein levels in humans. The majority of apoE2 homozygotes, who do not display overt type III hyperlipoproteinemia, have lower plasma cholesterol, low density lipoprotein (LDL) cholesterol, and apoB levels but higher plasma triglyceride and apoE levels than apoE3 homozygotes. In contrast, apoE4 is associated with higher plasma cholesterol, LDL cholesterol, and apoB levels but lower apoE levels (1–4). It also has been suggested that apoE4 is associated with a higher incidence of coronary heart disease (1, 4–9), probably due either to higher plasma cholesterol and LDL cholesterol levels or to potentially different roles of apoE isoforms within the lesions (5, 6, 10, 11). In contrast, apoE2 seems to reduce the risk for coronary heart disease, probably due to lower plasma LDL cholesterol levels, but only in subjects without type III hyperlipoproteinemia (1, 4, 7, 9). Those apoE2 homozygotes who develop type III hyperlipoproteinemia because of secondary precipitating factors are at increased risk for both coronary and peripheral artery atherosclerosis (1).

Although the cholesterol-lowering effect of apoE2 has been confirmed in several populations, the mechanism is not completely understood. One hypothesis is that defective LDL receptor binding of apoE2 lowers the transport of cholesterol-rich remnant particles into the liver, up-regulating hepatic LDL receptors and thereby accelerating the clearance of plasma LDL (1, 4). However, some experimental evidence shows that the number of hepatic LDL receptors is not increased in either human apoE2 (hE2) transgenic rabbits or apoE-null (mE−/−) mice (12, 13). Another hypothesis is that apoE2-containing very low density lipoproteins (VLDL) compete poorly with apoB-containing LDL for binding to the hepatic LDL receptor, speeding LDL clearance (13) and thereby decreasing plasma LDL. A third hypothesis is that apoE2 impairs lipolytic conversion of VLDL to LDL (14–17), perhaps by directly inhibiting lipoprotein lipase (LPL) activity (16).

Recently, we generated apoE2 transgenic mice in which moderate expression of apoE2 (10–30 mg/dl) on the wild-type mouse background produced a hypolipidemic phenotype, somewhat mimicking the hypolipolealemia in humans with apoE2; lower expression of apoE2 (~<10 mg/dl) did not alter plasma lipid levels significantly (18). Crossing hypolipidemic apoE2 mice (apoE2 ~<20 mg/dl) with LDL receptor-null (LDLR−/−) mice resulted in a typical type III hyperlipoproteinemic phenotype, characterized by increased levels of total cholesterol and triglycerides and by the accumulation of both apoE2 (50–60 mg/dl) and β-VLDL in plasma (19). These finding...
ings indicate that low LDL receptor number is one genetic factor responsible for converting apoE2-induced hyperlipidemia into an overt type III hyperlipoproteinemic phenotype. Furthermore, the LDL cholesterol levels were lower in the LDLR<sup>−/−</sup> mice expressing apoE2 than in the LDLR<sup>−/−</sup> mice, suggesting that the LDL cholesterol-lowering effect of apoE2 might occur even in the absence of the LDL receptor (19). However, this latter conclusion was tenuous because of the marked increase in remnant and intermediate density lipoproteins (IDL) in these mice.

In the present study, we evaluated in detail the potential role of the LDL receptor in apoE2-induced low LDL cholesterol by crossing transgenic mice expressing low levels of apoE2 (2–10 mg/dl) with LDLR<sup>−/−</sup> mice. Our data demonstrate that apoE2 consistently lowered the LDL cholesterol level in the absence of the LDL receptor. In vitro lipolytic studies indicated that low LDL cholesterol is caused by impairment of LPL-mediated lipolysis of triglyceride-rich lipoproteins. Furthermore, data obtained from hE2<sup>−/0</sup>,mE<sup>2</sup> mice demonstrated that the effects of apoE2 on plasma cholesterol and triglyceride levels are dose dependent and act via different mechanisms.

**EXPERIMENTAL PROCEDURES**

**Materials**—A Superox 6 column, purchased from Pharmacia (Uppsala, Sweden), was used on a Pharmacia fast protein liquid chromatography system. Centricron concentration filters were from Amicon (Lexington, MA). Cholesterol and triglyceride standards were from Abbott (North Chicago, IL) and Boehringer Mannheim (Indianapolis, IN), respectively. An automated system (Kinetic Microplate Reader, Molecular Devices, Menlo Park, CA) was used for lipid analysis. Bovine milk LP was from Sigma. Human apoC-II was provided by Dr. Karl H. Weisgraber (Glendale Institute of Cardiovascular Disease, San Francisco, CA). The LFS Lipogel assay kit was from Zaxis (Hudson, OH). All reagents for lipoprotein-agarose gels were from Ciba Corning (Palo Alto, CA). The ECL chemiluminescence detection kit for Western blots was from Amersham Life Science (Little Chalfont, Buckinghamshire, United Kingdom).

**Preparation of Transgenic Mice**—Hemizygous human apoE2 transgenic mice with endogenous mouse apoE (hE2<sup>+/+</sup>, ICR strain) were produced previously in our laboratory (18). LDLR<sup>−/−</sup> and mE<sup>2</sup> mice (both on 129Sv/J and B6D2F<sub>1</sub> strain) were purchased from Jackson Laboratories (Bar Harbor, ME). Female apoE2 mice from three low-expressing lines (apoE2 = 2, 5, and 10 mg/dl) were crossed with male LDLR<sup>−/−</sup> mice. The resulting obligate heterozygotes (LDLR<sup>−/−</sup>) expressing apoE2 (hE2<sup>−/0</sup>, LDLR<sup>−/−</sup>) were then crossed with LDLR<sup>−/−</sup> mice to yield apoE2 transgenic mice lacking LDL receptors (hE2<sup>−/0</sup>,LDLR<sup>−/−</sup>). Thus, the genetic background of these mice was 75% C57BL/6 and 25% ICR. To create apoE2 transgenic mice without endogenous apoE (hE2<sup>−/0</sup>,apoE<sup>−/−</sup>), female apoE2 mice from three different lines (apoE2 = 2, 10, and 20 mg/dl) were crossed with male mE<sup>2</sup> mice. The resulting obligate heterozygotes (mE<sup>−/−</sup>) expressing apoE2 (hE2<sup>−/0</sup>,mE<sup>−/−</sup>) were then crossed with mE<sup>−/−</sup> mice to yield apoE2 transgenic mice without mouse apoE (hE2<sup>−/0</sup>,mE<sup>−/−</sup>). Thus, the genetic background of the mice was also 75% C57BL/6 and 25% ICR.

The human apoE2 transgene was identified by immunoblotting of 1 µl of plasma with human-specific anti-apoE antisemur (18, 20). In the Western blot assay, human apoE2 was semiquantitated by comparing the densitometry readings of the sample bands with those of different concentrations of purified human apoE2. LDL receptor deficiency was assessed by polymerase chain reaction with specific primers designed according to the knockout gene construct. Mouse apoE deficiency was assessed by Western blotting with mouse-specific anti-apoE antisemur (19). All experiments were performed under protocols approved by the Committee on Animal Research, University of California, San Francisco.

**Lipoprotein Separation and Analysis**—Blood was collected from the tails of 8–12-week-old mice that had been fasted for 5 h. EDTA was used as anticoagulant (final concentration, 10 mM). Plasma was obtained by centrifugation at 14,000 rpm (microcentrifuge) for 10 min at 4 °C, and samples were stored for no more than 3 days at 4 °C in the presence of 1 mM phenylmethylsulfonyl fluoride as a protease inhibitor. Cholesterol and triglycerides were measured on total plasma and on chromatographic fractions by an enzymatic colorimetric method adapted for use with a microplate reader (21, 22).

Lipoproteins in 100 µl of plasma were separated by chromatography on a Superox 6 column, as described previously (18, 20, 22). The major lipoprotein classes eluted from the column were pooled and concentrated with Centricron filters (fractions 16–18, VLDL; fractions 19–22, IDL; fractions 23–27, LDL and a subclass of high density lipoproteins (HDL<sub>2</sub> and fractions 28–33, HDL). For agarose gel electrophoresis, 2-µl aliquots of concentrated lipoproteins were run on precast agarose gels (1%) for 45 min at 90 V. The gels were dried and stained with Fat Red 7B. In some cases, to analyze the distribution of apoE2 in various lipoproteins, the pooled samples representing different lipoprotein classes were separated on a 12% polyacrylamide-sodium dodecyl sulfate gel followed by immunoblotting with anti-human apoE antibodies. For analysis of chemical compositions and lipolysis of VLDL, the VLDL (d < 1.006 g/ml) were isolated from plasma by ultracentrifugation at 98,000 rpm for 2 h at 4 °C in a Beckman TL100 ultracentrifuge (23). ApoE2-proteins were separated on 3–20% polyacrylamide-sodium dodecyl sulfate gradient gels. The amounts of human apoE2, mouse apoE, or mouse apoC-II were determined by Western blotting with polyclonal antibodies against human apoE, mouse apoE, or mouse apoC-II, respectively. Purified human apoE2, mouse apoE, and mouse apoC-II were used as standards, respectively.

Alternatively, lipoproteins in 8 µl of plasma were separated by LFS Lipogel electrophoresis (Zaxis, Hudson, OH), fractionated according to their particle size in a 0.5–30% polyacrylamide gradient gel, and stained for cholesterol. The distribution of cholesterol in various lipoproteins was determined by densitometry at 600 nm. The cholesterol levels in various lipoproteins were calculated from the plasma total cholesterol determined by an enzymatic colorimetric method. For five normal human plasma standards, the intra-assay coefficients of variation were 9.8, 5.3, and 6.4% for VLDL/LDL, HDL cholesterol, respectively; the inter-assay coefficients of variation were 14.2, 7.1, and 8.3%, respectively.

**Lipolysis of VLDL and IDL in Vitro**—To determine the ability of normal and apoE2 VLDL to serve as substrates for lipase-mediated lipolysis, 30 µg of VLDL or IDL triglyceride was incubated for 30 min at 37 °C with 10 µl of VLDL-depleted postheparin mouse plasma, which was collected from normal mice 10 min after intravenous injection of heparin (50 units/kg). The incubation was performed in the presence of 1.2 m NaCl (to measure hepatic lipase) or in its absence (to measure total lipolytic activity). In some cases, specific amounts of purified human apoC-II were included in the incubation. After incubation, the levels of released free fatty acids were determined by an enzymatic colorimetric method (24) (Wako Chemicals, Richmond, VA). The LPL-mediated lipolysis was calculated as the difference between total lipolytic activity and lipolytic activity in the presence of apoC-II.

**Lipolysis of Triglyceride-rich Emulsion Particles in Vitro**—The triglyceride-rich emulsion particles were prepared as described previously (26–28). Briefly, 100 mg of triolein (Sigma) and 25 mg of egg yolk phosphatidylcholine were mixed together and dried under nitrogen. The pellets were resuspended in 5 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 100 mM KC1 and then sonicated. The resulting emulsion particles were similar in size to native human VLDL (27, 29). To determine the effect of apoE2 on LPL-mediated lipolysis, the triglyceride-rich emulsion particles (50 µl) were incubated at 37 °C first with 4 µg of human apoC-II for 30 min and then with different amounts of apoE2 and 0.5 µg of bovine milk LPL (Sigma) for 30 min. To determine the effect of apoC-II on LPL-mediated lipolysis, the triglyceride-rich emulsion particles (50 µl) were incubated at 37 °C first with 4 µg of human apoC-II for 30 min and then with different amounts of apoC-II and 0.5 µg of bovine milk LPL (Sigma) for 30 min. After incubation, the lipolytic activity was determined as described above.

**Statistical Analysis**—Mean lipid levels are reported as the mean ± S.D. Differences in lipid levels were evaluated by t test. Correlations of plasma apoE2 and lipid levels were assessed by regression analysis.

**RESULTS**

**Effects of ApoE2 Expression on Plasma Lipids and Lipoproteins in the Absence of LDL Receptors**—To generate LDLR<sup>−/−</sup> mice expressing different levels of apoE2, we created LDLR<sup>−/−</sup> mice with the low dose expression level of apoE2. Table I summarizes the plasma levels of lipids and lipoproteins in hE2<sup>−/0</sup>,LDLR<sup>−/−</sup> and hE2<sup>300</sup>,LDLR<sup>−/−</sup> mice. As reported previously (20), both male and female LDLR<sup>−/−</sup> mice had significant hypercholesterolemia (total cholesterol levels about double those of nontransgenic controls) and slightly increased triglyceride levels (25% higher than in nontransgenic controls) (data

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*Note: The text contains citations which are not shown here.*
not shown for nontransgenic mice). Gel filtration chromatography on a Superose 6 column (Fig. 1A) and nondenaturing polyacrylamide gradient gel electrophoresis (Fig. 2) demonstrated that the hypercholesterolemia in the LDLR−/− mice was due mainly to an accumulation of LDL cholesterol.

The hE2<sup>−/−</sup>,LDLR−/− mice expressing apoE2 at <10 mg/dl had lower total cholesterol (9 and 7% for males and females, respectively) than hE2<sup>0/0</sup>,LDLR−/− mice (Table I). The decrease was due to reductions of about 15% in both LDL and HDL cholesterol, which were partially offset by an increase in VLDL/IDL cholesterol (males, 39%; females, 30%) (Table I, Fig. 1B, and Fig. 2). Plasma triglyceride levels were higher in both males (32%) and females (42%) than in hE2<sup>0/0</sup>,LDLR−/− mice (Table I). Mice expressing apoE2 at 10–20 mg/dl had a greater decrease in total cholesterol (14 and 13% for males and females, respectively) than hE2<sup>0/0</sup>,LDLR−/− mice (Table I). Again, the decrease was due to reductions of both LDL (males, 32%; females, 29%) and HDL (males, 24%; females, 19%) cholesterol; however, the hE2<sup>−/−</sup>,LDLR−/− mice had higher VLDL/IDL cholesterol (males, 64%; females, 40%) (Table I, Fig. 1C, and Fig. 2). Plasma triglyceride levels were increased significantly in both male (62%) and female (85%) mice (Table I), and triglyceride-enriched LDL appeared (Fig. 1C). Mice expressing apoE2 at more than 20 mg/dl had an even greater decrease in total cholesterol (males, 20%; females, 17%) but more severe hypertriglyceridemia (males, 131%; females, 167%) than hE2<sup>0/0</sup>,LDLR−/− mice (Table I). Their LDL and HDL cholesterol levels were about half of those in hE2<sup>0/0</sup>,LDLR−/− mice (Table I); most significantly, the distribution of plasma cholesterol was shifted predominantly to VLDL/IDL fractions (Fig. 1D and Fig. 2). Thus, in general, there was a strong negative correlation between plasma apoE2 concentrations and plasma total cholesterol levels (males, r = −0.81, p < 0.001; females, r = −0.87, p < 0.001) (Fig. 3A). Likewise, there was a negative correlation between apoE2 and LDL cholesterol in females, r = −0.89, p < 0.001; females, r = −0.72, p < 0.001) or HDL cholesterol (males, r = −0.72, p < 0.001; females, r = −0.81, p < 0.001) (data not shown). On the other hand, there was a strong positive correlation between plasma apoE2 concentrations and VLDL/IDL cholesterol (males, r = 0.68, p < 0.001; females, r = 0.78, p < 0.001) and plasma triglyceride levels (males, r = 0.74, p < 0.001; females, r = 0.84, p < 0.001) (Fig. 3B). We have previously shown that hE2<sup>−/−</sup>,LDLR−/− mice expressing 50–60 mg/dl apoE2 have a hypercholesterolemia characterized by a marked increase in β-VLDL (19).

Column fractions representing various lipoproteins were pooled, concentrated, and subjected to agarose gel electrophoresis (Fig. 4). As expected, hE2<sup>0/0</sup>,LDLR−/− mice accumulated a large amount of β-migrating LDL (Fig. 4A). The hE2<sup>−/−</sup>,LDLR−/− mice expressing <20 mg/dl apoE2 had a significant decrease in LDL and a slight increase in VLDL (Fig. 4B). In mice expressing >20 mg/dl apoE2, the decrease in LDL was more pronounced, and the VLDL increased dramatically and migrated more slowly toward the β-position (Fig. 4C). Taken together, all the above data demonstrate that apoE2 has an LDL cholesterol-lowering effect independent of the LDL receptor in transgenic mice.

**Distribution of ApoE2 among Lipoproteins**—The distribution of apoE2 among various lipoprotein classes is shown in Table [1](#). The abbreviations are the following: hE2, human apoE2; TC, total cholesterol; TG, triglyceride; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; VLDL/IDL-C, VLDL/IDL cholesterol. Differences were evaluated by t test.

|                | hE2<sup>−/−</sup>,LDLR−/− | hE2<sup>0/0</sup>,LDLR−/− |
|----------------|---------------------------|---------------------------|
| **Male**       |                           |                           |
| n              | 8                         | 11                        | 11                        |
| ApoE2          | 0                         | 6 ± 2                     | 15 ± 3                    |
| TC             | 219 ± 13                  | 199 ± 19<sup>a</sup>      | 189 ± 12<sup>ab</sup>     |
| TG             | 74 ± 20                   | 98 ± 29                   | 120 ± 31<sup>a</sup>      |
| VLDL/IDL-C     | 36 ± 10                   | 50 ± 6<sup>b</sup>        | 59 ± 8<sup>b</sup>        |
| LDL-C          | 99 ± 7                    | 83 ± 6<sup>b</sup>        | 67 ± 6<sup>b</sup>        |
| HDL-C          | 83 ± 5                    | 70 ± 6<sup>b</sup>        | 63 ± 4<sup>b</sup>        |
| **Female**     |                           |                           |
| n              | 9                         | 7                         | 9                         |
| ApoE2          | 0                         | 6 ± 2                     | 14 ± 2                    |
| TC             | 209 ± 11                  | 195 ± 14<sup>a</sup>      | 182 ± 9<sup>ab</sup>      |
| TG             | 67 ± 17                   | 95 ± 17<sup>a</sup>       | 124 ± 24<sup>ab</sup>     |
| VLDL/IDL-C     | 40 ± 4                    | 52 ± 4                    | 56 ± 6                    |
| LDL-C          | 97 ± 7                    | 83 ± 4                    | 69 ± 6                    |
| HDL-C          | 72 ± 6                    | 62 ± 4                    | 58 ± 5                    |

<sup>a</sup> p < 0.05 versus hE2<sup>0/0</sup>,LDLR−/− mice.  
<sup>b</sup> p < 0.001 versus hE2<sup>0/0</sup>,LDLR−/− mice.
tors. Previously, we showed that high levels of apoE2 on the
mas from LDLR
apoE2 levels
to a direct effect of apoE2 on lipolytic processing. At
mg/dl) (Fig. 3
having no apoE at all and that increasing the amount of apoE2
levels in the hE2
apoE2 distribution pattern in between those of mice expressing
apoE2 in the VLDL fraction than the mice expressing apoE2 at
less apoE2 in the HDL fraction but almost three times more
apoE2-containing VLDL and IDL are not good substrates for LPL-mediated lipolysis compared with normal
The apoE2-containing VLDL from hE2
apoE2 mice contained much more cholesterol than normal VLDL, which was reflected by a much lower ratio of triglyceride to cholesterol (Table III). To determine if the higher cholesterol content of apoE2-containing VLDL, rather than apoE2 itself, is responsible for the impaired lipolysis, we isolated hE2
apoE2 mice. We compared the LPL-mediated lipolysis of hE2
apoE2 mice (Fig. 5). The presence of apoE2 in the VLDL and IDL from the hE2
apoE2 mice inhibited LPL-mediated lipolysis by 84 and 73%, respectively, compared with VLDL and IDL from nontransgenic mice, while hepatic lipase-mediated lipolysis appeared to be affected to a lesser degree. These results indicate that apoE2-containing VLDL and IDL are not good substrates for LPL-mediated lipolysis compared with normal VLDL. The VLDL and IDL from LDLR
apoE2 mice were, however, good substrates for LPL.

Effect of ApoE2 Expression on Plasma Lipids in the Absence of Endogenous Mouse ApoE—The positive correlation between plasma apoE2 and triglyceride levels in the hE2
apoE2 mice mimics a condition observed in humans (31, 32). However, because hE2
apoE2 mice lack LDL receptors, it is difficult to determine whether increased plasma triglyceride levels are caused solely by an increase in apoE2 or by an increase in apoE2 in combination with the absence of LDL receptors. To address this question, we generated apoE2 transgenic mice on the mE
apoE2 mice expressing low and intermediate levels of apoE2, which is an absolute cofactor for LPL activity (37, 38). To evaluate this possibility, we added purified human apoC-II to apoE2-expressing mice. Since previous studies had shown that increasing apoE in VLDL displaces apoCs from the particles (33), and vice versa (33–36), the impairment of VLDL lipolysis by apoE2 could occur via a displacement or masking of apoC-II, which is an absolute cofactor for LPL activity (37, 38). To evaluate this possibility, we added purified human apoC-II to various VLDL and determined its effect on lipolysis (Fig. 5A). Adding apoC-II to apoE2-containing VLDL stimulated LPL-mediated lipolysis 3-fold, but did not significantly affect the lipolysis of normal VLDL. As expected, apoC-II did not affect hepatic lipase-mediated lipolysis of either normal or apoE2-containing VLDL (Fig. 5A). Similar results were also obtained from various IDL (Fig. 5B). These data indicate that apoE2 impaired lipolysis of VLDL can be partially corrected by increasing the amount of apoC-II on the particles. The addition of apoC-II to nontransgenic or LDLR
apoE2 VLDL or IDL had only a very small stimulatory effect.

Since adding apoE2 to normal VLDL inhibited lipolysis in a dose-dependent fashion (data not shown), it is probable that
both plasma cholesterol and triglycerides (Fig. 3, C and D). These data indicate that the effects of apoE2 on plasma cholesterol and triglyceride levels are dose dependent and may act via different mechanisms.

Effect of ApoE2 on Lipolysis of VLDL and IDL—The simultaneous increase in plasma triglycerides and VLDL/IDL cholesterol and the decrease in LDL cholesterol in the presence of an increasing level of apoE2 in hE2
apoE2 mice raise the possibility that apoE2 impairs the lipolytic conversion of VLDL to LDL, as previously hypothesized (16). To address this question, we determined the ability of normal and apoE2-containing VLDL and IDL to serve as substrates for lipase-mediated lipolysis in vitro (Fig. 5). The presence of apoE2 in the VLDL and IDL from the hE2
apoE2 mice inhibited LPL-mediated lipolysis by 84 and 73%, respectively, compared with VLDL and IDL from nontransgenic mice, while hepatic lipase-mediated lipolysis appeared to be affected to a lesser degree. These results indicate that apoE2-containing VLDL and IDL are not good substrates for LPL-mediated lipolysis compared with normal VLDL. The VLDL and IDL from LDLR
apoE2 mice were, however, good substrates for LPL.

Effect of the ApoE2:ApoC-II Ratio on the Lipolysis of VLDL and Triglyceride-rich Emulsion Particles—Another difference in the VLDL composition was that apoE2-containing VLDL had much more apoE, but much less apoC-II, than normal VLDL (Table III). The apoC-II content of the d < 1.006 g/ml lipoproteins was 5–6-fold higher in nontransgenic mice than in apoE2-expressing mice. Since previous studies had shown that increasing apoE in VLDL displaces apoCs from the particles (33), and vice versa (33–36), the impairment of VLDL lipolysis by apoE2 could occur via a displacement or masking of apoC-II, which is an absolute cofactor for LPL activity (37, 38). To evaluate this possibility, we added purified human apoC-II to various VLDL and determined its effect on lipolysis (Fig. 5A). Adding apoC-II to apoE2-containing VLDL stimulated LPL-mediated lipolysis 3-fold, but did not significantly affect the lipolysis of normal VLDL. As expected, apoC-II did not affect hepatic lipase-mediated lipolysis of either normal or apoE2-containing VLDL (Fig. 5A). Similar results were also obtained from various IDL (Fig. 5B). These data indicate that apoE2-impaired lipolysis of VLDL can be partially corrected by increasing the amount of apoC-II on the particles. The addition of apoC-II to nontransgenic or LDLR
apoE2 VLDL or IDL had only a very small stimulatory effect.

Since adding apoE2 to normal VLDL inhibited lipolysis in a dose-dependent fashion (data not shown), it is probable that
the apoE2:apoC-II ratio is a major factor that modulates the lipolysis. To evaluate the importance of this ratio as a determinant of lipolysis, VLDL-like triglyceride-rich emulsion particles were prepared with increasing amounts of apoE2 and a constant amount of apoC-II. Increasing the apoE2:apoC-II ratio progressively inhibited lipolysis, reaching 90% at a ratio of 3.0 (Fig. 6A). In an additional set of experiments, we reisolated the particles after the addition of the apoproteins. We found that at lower levels of added apoE2, the apoE2:apoC-II ratio was only slightly lower than predicted, and at higher levels of added apoE2, the apoE2:apoC-II ratio was higher than predicted. At the highest level of added apoE2, where lipolysis was inhibited.

**FIG. 3.** Correlation of plasma apoE2 and lipids. A, apoE2 versus cholesterol in hE2<sup>−/−</sup>LDLR<sup>−/−</sup> mice. B, apoE2 versus triglycerides in hE2<sup>−/−</sup>LDLR<sup>−/−</sup> mice. C, apoE2 versus cholesterol in hE2<sup>−/−</sup>LDLR<sup>−/−</sup> mice. D, apoE2 versus triglycerides in hE2<sup>−/−</sup>LDLR<sup>−/−</sup> mice.

**FIG. 4.** Agarose gel electrophoresis of lipoprotein fractions from male LDLR<sup>−/−</sup> mice expressing different levels of apoE2. The VLDL are Superose 6 fractions 16–18, and the IDL are fractions 19–22. The origin and migration positions of α-migrating (HDL), pre-β-migrating (VLDL), and β-migrating (LDL) lipoproteins are indicated.

**TABLE II**

| ApoE2 | VLDL | IDL | LDL/HDL<sub>1</sub> | HDL |
|-------|------|-----|-------------------|-----|
| mg/dl |      |     |                   |     |
| <10   | 9 ± 2| 7 ± 1| 24 ± 2            | 60 ± 4|
| 10–20 | 16 ± 2| 9 ± 2| 25 ± 3            | 50 ± 3|
| >20   | 23 ± 3| 9 ± 2| 27 ± 3            | 41 ± 3|

**FIG. 5.** Effect of apoE2 and apoC-II on the lipolysis of VLDL and IDL. Various VLDL (A) or IDL (B) (30 μg of triglycerides) were incubated with 10 μl of VLDL-depleted postheparin mouse plasma for 30 min at 37 °C with or without 1.2 M NaCl. In some cases, various VLDL or IDL (30 μg of triglycerides) were first incubated with 8 μg of apoC-II for 30 min at 37 °C and then with 10 μl of VLDL-depleted postheparin mouse plasma for 30 min at 37 °C with or without 1.2 M NaCl. Lipase activities were calculated as described under “Experimental Procedures.” For the apoE2 content of the various VLDL, see Table III. Results are the mean ± S.D. of determinations in four mice. FFA, free fatty acid.
Apoproteins of VLDL pooled from 3 to 5 mice were separated on 3–20% polyacrylamide–sodium dodecyl sulfate gradient gels. The amounts of human apoE2 (hApoE2), mouse apoE (mApoE), or mouse apoC-II (mApoC-II) were determined by Western blotting with polyclonal antibodies against human apoE, mouse apoE, or mouse apoC-II, respectively. TC, total cholesterol; TG, triglyceride.

| VLDL | TG/TC | hApoE2 | mApoE | mApoC-II |
|------|-------|--------|-------|----------|
| Nontransgenic | 14.6 | 0 | 19 | 32 |
| hE20/0,LDLR−/− | 9.4 | 0 | 30 | 26 |
| hE20/0,mE−/− | 1.2 | 51 | 11 | 5 |
| hE20/0,mE−/− | 0.2 | 0 | 0 | 38 |
| hE20/0,mE−/− | 1.2 | 55 | 0 | 6 |

FIG. 6. Effect of the interaction of apoE2 with apoC-II on LPL-mediated lipolysis of triglyceride-rich emulsion particles. A, 5 μl of triglyceride-rich emulsion particles was incubated first with 4 μg of apoC-II for 30 min at 37 °C and then with 10 μl of bovine milk LPL (0.5 μg protein) and different amounts of apoE2 for 30 min at 37 °C. B, 50 μl of triglyceride-rich emulsion particles was incubated first with 4 μg of apoE2 for 30 min at 37 °C and then with 10 μl of bovine milk LPL (0.5 μg of protein) and different amounts of apoC-II for 30 min at 37 °C. After incubations in both experiments (A and B), LPL activity was calculated as described under “Experimental Procedures.” Results are the mean ± S.D. of determinations in triplicate experiments. FFA, free fatty acid.

FIG. 6B. Characteristic of VLDL (d < 1.006 g/ml) from various transgenic mice

ApoE2/LDL Receptor-null Mice

The LDL cholesterol-lowering effect of apoE2 has been offered as the explanation for reduced risk for coronary heart disease in subjects with apoE2 who do not have type III hyperlipoproteinemia (1–4, 7, 9). Thus, defining the mechanism of this phenomenon will clarify the role of different apoE isoforms in plasma lipoprotein metabolism and in the development of atherosclerosis. Since up-regulation of hepatic LDL receptors due to a delayed liver uptake of apoE2-containing remnant lipoproteins has long been suggested to explain the LDL cholesterol-lowering effect of apoE2 (4), we crossed transgenic mice expressing low levels of apoE2 (2–10 mg/dl) with LDLR−/− mice to evaluate the involvement of the LDL receptor in this phenomenon. Our data demonstrating that apoE2 lowers LDL cholesterol in apoE2 transgenic mice lacking LDL receptors suggests that there is a mechanism independent of the LDL receptor to explain this phenomenon. Evidence from other studies supports this conclusion: neither apoE2 transgenic rabbits nor mE−/− mice have more hepatic LDL receptors than nontransgensics (12, 13). Previously, apoE2 has been shown to impair the lipolytic conversion of VLDL to LDL (14–17). Our present studies further indicate that apoE2 lowers LDL cholesterol by impairing the lipolysis of triglyceride-rich lipoproteins both in vivo and in vitro.

High density lipoprotein levels in humans homozygous for apoE2 are usually low, especially in type III hyperlipoproteinemic subjects (4, 39). Impairment of LDL lipolysis by apoE2 provides a logical explanation for the HDL-lowering effect in apoE2 transgenic mice on the wild-type, mE−/−, or LDLR−/− background (18, 19). Since the formation of at least a portion of HDL particles requires the surface components of triglyceride-rich lipoproteins released during lipolytic processing (40), apoE2-associated impairment of lipolysis would lead to a lesser release of surface components from the triglyceride-rich lipoproteins, which in turn would decrease the production of HDL particles. This possibility is supported by the observation that patients with homozygous LPL deficiency have extremely high levels of chylomicrons and VLDL and reduced levels of LDL and HDL (41). Hypertriglyceridemia with reduced LDL and HDL can also be induced by infusing antibodies that inhibit LPL into monkeys (42). In addition, plasma LPL activity correlates positively with HDL cholesterol levels (43).

Population studies have demonstrated that apoE2 is associated with increased plasma triglyceride levels (31, 32), which is confirmed in this study. This phenomenon has been ascribed to defective LDL receptor binding of apoE2, which impairs the clearance of triglyceride-rich lipoproteins from the circulation (1, 32). However, the normal or only mildly elevated triglyceride levels in mE−/− mice with severe hypercholesterolemia argues against this hypothesis (44, 45). The present studies of the hE20/0,LDLR−/− mice provide evidence supporting the hypothesis that apoE2-induced hypertriglyceridemia is caused not only by impaired remnant lipoprotein clearance but also to a major extent by impaired lipolysis of triglyceride-rich lipoproteins (1, 32). Likewise, our previous studies (19) and the present expanded studies demonstrate that moderate and high expression of apoE2 on the mE−/− background (hE20/0, mE−/−) increased plasma triglyceride levels 2–5-fold above those in mE−/− mice (Fig. 3D), indicating that as far as triglyceride catabolism is concerned, having defective apoE2 is worse than having no apoE at all. On the other hand, since plasma total cholesterol levels were lower in the hE20/0, mE−/− than in the mE−/− mice (Fig. 3C and Ref. 19), it seems that the hypercholesterolemia and hypertriglyceridemia associated with apoE2 occur via two separate mechanisms: hypercholesterolemia caused by defective binding of apoE2 to the LDL receptor and hypertriglyceridemia caused mostly by impaired lipolysis of triglyceride-rich lipoproteins by apoE2.

The present study also shows that the apoE2:apoC-II ratio in VLDL particles affects lipolysis. Increasing this ratio in VLDL or triglyceride-rich emulsion particles impaired LPL-mediated lipolysis. Conversely, decreasing this ratio in these apoE2-containing particles corrected or enhanced LPL-mediated lipolysis. Thus, it seems that apoE2-containing VLDL are poor substrates for lipolysis partly because of displacement of apoC-II, although a direct effect of apoE2 on lipolysis cannot be excluded (46, 47). Taken together with data showing that the apoE:apoC ratio is also an important determinant for apoE-mediated clearance of remnant lipoproteins via the hepatic receptors (33–36), our present data indicate that the apoE2:
apoC-II ratio is also an important determinant for LPL-mediated lipolysis of triglyceride-rich lipoproteins. Thus, as remnant lipoproteins accumulate in the plasma, the level of apoE2 in the remnant fraction increases, resulting in impaired lipolysis and a blockade in the VLDL → LDL lipolytic cascade. We should point out that displacement of apoC-II and impairment of lipolysis by apoE2 is not specific for this isoform. In vitro, all three apoE isoforms inhibit lipolysis to a similar extent. Thus, under the right genetic and physiological conditions, high plasma levels of any of the apoE isoforms might have severe consequences. However, these consequences most likely manifest themselves in the presence of the apoE2 isoform, since this is the isoform that accumulates significantly in the plasma because it binds defectively to lipoprotein receptors.

Based on these studies and our previous studies (19) with increasing concentrations of apoE2 expression on the mE/−/− background, we have defined how apoE2 modulates both cholesterol and triglyceride levels in mice. We have shown that the effects of apoE2 on both cholesterol and triglyceride levels are dose dependent and act via different mechanisms. Low expression of apoE2 (4–8 mg/dl) in the hE2+/−,mE/−/− mice does not provide enough apoE2 molecules to the remnant fractions to mediate clearance of the particles (because of the defective receptor binding of apoE2), leading to only a slight reduction of the hypercholesterolemia of the mE/−/− mice. Furthermore, low levels of apoE2 in VLDL or remnant lipoproteins are not sufficient to displace or mask the apoC-II; thus, the lipolysis is effective and plasma triglyceride levels are normal. However, intermediate levels of apoE2 (15–25 mg/dl) at least partially compensate for the absence of normal apoE by overloading the particles with apoE2, which even though partially defective, can mediate receptor binding and remnant clearance. Thus, cholesterol levels decrease toward normal. However, triglyceride levels begin to rise sharply as the increased apoE2 displaces or masks the apoC-II and impairs LPL-mediated lipolysis of VLDL and IDL. At high levels of apoE2 expression (>25 mg/dl), the remnants become saturated with apoE2, markedly impairing LPL-mediated lipolysis and remnant clearance and leading to severe hypertriglyceridemia and hypercholesterolemia. Both the incomplete lipolysis of the remnants and the defective receptor-mediated clearance combine to result in the severe hyperlipidemia characterizing type III hyperlipoproteinemia.

Similarly, human apoE2 homozygotes who are hypolipidemic have low levels of LDL cholesterol and only a slight accumulation of remnant lipoprotein cholesterol and triglycerides (Fig. 7). Presumably the low plasma levels of apoE2 mimic what is seen in the intermediate-expressing hE2+/−,mE/−/− mice, i.e. the apoE2 level is sufficient to mediate at least some remnant clearance either by the LDL receptor (even though apoE2 has defective binding) or by the heparan sulfate proteoglycan/LDL receptor-related protein pathway (apoE2 binding is fairly normal), leading to only a small increase in remnant lipoproteins. However, overt type III hyperlipoproteinemia with marked hypercholesterolemia and hypertriglyceridemia can be precipitated when other factors are present, such as a high rate of VLDL production, a low number of LDL receptors as demon-

\[\text{apoE2/LDL Receptor-null Mice}\]

**Fig. 7. Receptor-mediated clearance and lipolytic processing: VLDL \rightarrow LDL.** In normolipidemic subjects, apoE3 plays a key role in mediating the clearance of remnant lipoproteins from plasma by the liver. In hypolipidemic apoE2 homozygotes, the plasma levels of apoE2 are increased due to its defective receptor binding. These increased levels may compensate for apoE2's defective receptor binding and allow it to mediate efficient remnant clearance from the plasma by the LDL receptor (even though apoE2 has defective binding) or by the heparan sulfate proteoglycan/LDL receptor-related protein pathway (apoE2 binding is fairly normal), leading to only a small increase in remnant lipoproteins. However, these increased levels of apoE2 are sufficient to displace or mask apoC-II and impair lipolytic processing of VLDL, leading to decreased LDL formation and decreased LDL levels. In the presence of secondary factors, the already compromised remnant catabolism in the apoE2/2 homozygotes is further stressed, resulting in a further impairment of clearance and an accumulation of apoE2. The enrichment of remnant lipoproteins with apoE2 would severely displace or mask the apoC-II, further impairing lipolysis and disrupting remnant clearance. CH, apoC-II; E2, apoE2; E3, apoE3. Dashed lines indicate blockage of either LDL formation or remnant clearance.

\[\text{apoE2/LDL Receptor-null Mice}\]

\[\text{ApoE3/3 Normolipidemic}\]

\[\text{Liver Uptake}\]

\[\text{Remnant-Cholesterol} \quad \text{Remnant-Triglycerides} \quad \text{Remnant-ApoE2} \quad \text{LDL Cholesterol}\]

\[\text{Normal} \quad \text{Normal} \quad \text{—} \quad \text{Normal}\]

\[\text{ApoE2/2 Hypolipidemic}\]

\[\text{Liver Uptake}\]

\[\text{ApoE2/2 Hyperlipidemic}\]

\[\text{Liver Uptake}\]

\[\text{LDL Cholesterol}\]

\[\uparrow \quad \uparrow \quad \downarrow \quad \downarrow\]

\[\text{Y. Huang, X. Q. Liu, S. C. Rall, Jr., and R. W. Mahley, unpublished observations.}\]
strated in this and a previous study (19), or low estrogen status, which decreases both LDL receptor numbers and lipolytic activities (12). These factors further stress the already compromised remnant catabolism in the apoE2 homozygotes, resulting in a further impairment of clearance and lipolytic processing and an accumulation of apoE2. As with the high-expressing hE2+/+mE−/− mice, the enrichment of the remnant lipoproteins with apoE2 would severely displace or mask the

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