Molecular Mechanisms of Type III Hyperlipoproteinemia: The Contribution of the Carboxy-Terminal Domain of ApoE Can Account for the Dyslipidemia That Is Associated with the E2/E2 Phenotype†

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ABSTRACT: Apolipoprotein E2, which has an R158 for C substitution, has reduced affinity for the LDL receptor and is associated with type III hyperlipoproteinemia in humans. Consistent with these observations, we have found that following adenovirus-mediated gene transfer, full-length apoE2 aggravates the hypercholesterolemia and induces hypertriglyceridemia in E-deficient mice and induces combined hyperlipidemia in C57BL/6 mice. Unexpectedly, the truncated apoE2−202 form that has an R158 for C substitution when expressed at levels similar to those of the full-length apoE2 normalized the cholesterol levels of E-deficient mice without induction of hypertriglyceridemia. The apoE2 truncation increased the affinity of POPC−apoE particles for the LDL receptor, and the full-length apoE2 had a dominant effect in VLDL triglyceride secretion. Hyperlipidemia in normal C57BL/6 mice was prevented by coinfection with equal doses of each, the apoE2 and the apoE2−202-expressing adenoviruses, indicating that truncated apoE forms have a dominant effect in remnant clearance. Hypertriglyceridemia was completely corrected by coinfection of mice with an adenovirus-expressing wild-type lipoprotein lipase, whereas an inactive lipoprotein lipase had a smaller effect. The findings suggest that the apoE2-induced dyslipidemia is not merely the result of substitution of R158 for C but results from increased secretion of a triglyceride-enriched VLDL that cannot undergo lipolysis, inhibition of LpL activity, and impaired clearance of chylomicron remnants. Infection of $E^{-/-}$xLDLr$^{-/-}$ double-deficient mice with apoE2−202 did not affect the plasma cholesterol levels, and also did not induce hypertriglyceridemia. In contrast, apoE2 exacerbated the hypercholesterolemia and induced hypertriglyceridemia, suggesting that the LDL receptor is the predominant receptor in remnant clearance.

Familial type III hyperlipoproteinemia (HLP)[1] is characterized by xanthomas, elevated cholesterol, triglyceride, and apoE levels, and premature atherosclerosis (*1*, *2*).

Studies in human patients with type III hyperlipoproteinemia and in animal models with apoE deficiency or defective apoE forms (*3*−*11*) showed that apoE is required for the clearance of cholesterol ester-rich lipoprotein remnants found in the VLDL and IDL region (*3*, *5*, *8*−*11*). The accumulation of such remnants in plasma is responsible for the development of atherosclerosis (*3*, *5*−*7*). ApoE may also be involved in cholesterol efflux processes (*12*−*14*), and these functions of apoE contribute to cell and tissue cholesterol homeostasis (*12*−*14*) and may explain why, when expressed locally in macrophages or endothelial cells, apoE protects against atherosclerosis (*15*−*17*).

Previous studies had shown that the catabolism of ^{125}I-labeled apoE derived from an individual with type III HLP having the E2/2 phenotype was slower than catabolism of apoE derived from normal subjects (*18*, *19*). In addition, *in vitro* studies have shown that apoE from individuals with the E3/3 and E4/4 phenotypes displays the same competition for the LDL receptor, whereas apoE derived from individuals with the E2/2 phenotype competes less efficiently (*20*). Previous studies also indicated that in most cases type III HLP is expressed in families with a tendency to hypertriglyceridemia that may be caused by environmental or other genetic factors, some combination thereof, or both (*21*).

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[1] Abbreviations: apoE, apolipoprotein E; apoA-I, apolipoprotein A-I; apoCII, apolipoprotein CII; Ad, adenovirus; BSA, bovine serum albumin; ELISA, enzyme-linked immunoassay; FBS, fetal bovine serum; FPLC, fast pressure liquid chromatography; GAPDH, glyceride acid phosphate dehydrogenase; GFP, green fluorescence protein; HDL, high-density lipoprotein; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; VLDL, very low-density lipoprotein; LDLr, low-density lipoprotein receptor; HLP, familial type III hyperlipoproteinemia; LpL, lipoprotein lipase.

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We have shown recently that overexpression of full-length apoE forms in apoE-deficient mice ($E^{-/-}$) is associated with high cholesterol and triglyceride levels, whereas overexpression of truncated apoE forms apoE4−185, apoE4−202, apoE4−229, and apoE4−259 normalizes the cholesterol levels of apoE-deficient mice and does not trigger hypertriglyceridemia (*22*−*24*). Full-length apoE4 greatly increased hepatic VLDL triglyceride secretion, whereas the truncated apoE4−202 and apoE4−185 did not have a significant effect on VLDL triglyceride secretion (*23*, *24*). Overall, these findings indicated that amino-terminal residues 1−185 of apoE are sufficient to promote binding to lipoprotein remnants and direct their clearance by lipoprotein receptors *in vivo*, whereas the carboxy-terminal region (residues 260−299) of apoE contributes to hypertriglyceridemia.

The study presented here had two major objectives: (1) to determine the contribution of the R158 for C substitution to the induction of high cholesterol and triglyceride levels in the carriers of the E2/2 phenotype and to elucidate further the mechanism of apoE-induced hypertriglyceridemia and (2) to assess the role of lipoprotein receptors other than the LDL receptor in the hepatic clearance of apoE-containing lipoproteins. Our findings suggest that the inefficient clearance of the lipoprotein remnants observed in type III HLP patients with the apoE2/2 phenotype may not be the result of the R158 for C substitution but is brought about by carboxy-terminal residues 203−299 of apoE. It appears that the carboxy-terminal 203−299 region of apoE decreases the affinity of apoE-containing lipoprotein particles for the LDL receptor, and directly or indirectly hinders lipolysis and receptor uptake of triglyceride-rich lipoproteins. This study also indicated that truncated apoE forms have a dominant effect in remnant clearance and that the LDL receptor is the predominant receptor that contributes significantly to remnant clearance.

EXPERIMENTAL PROCEDURES

Generation of Adenoviral Constructs. The construction of the apoE4- and apoE4−202-expressing adenoviruses has been described previously (*24*). For the construction of the apoE2−202-expressing adenovirus, the *Eco*RI fragment of the human apoE2 gene, which includes the entire exon IV sequence, was cloned into the *Eco*RI site of the pBS vector to generate the vector pBlue-E2-exIV_g. The pUC-apoE2−202 plasmid was generated by overlap-extension PCR that resulted in mutagenesis of codon 203 (GTA) to a stop codon (TAA). The pUC-apoE2−202 plasmid was digested with *Sty*I and *Bbs*I, and the mutated sequence was exchanged for the wild-type (WT) sequence of the pBlue-E2-exIV_g plasmid to generate the pBlue-E2−202-exIV_g plasmid.

The recombinant viruses were constructed as described previously (*24*) using the Ad-Easy-1 system where a recombinant adenovirus construct is generated in bacteria BJ-5183 cells (*25*). Correct clones were propagated in RecA DH5α cells. The recombinant adenoviral vectors were linearized with *Pac*I and used to infect 911 cells (*26*). Following large-scale infection of cell cultures, the recombinant adenoviruses were purified by two consecutive CsCl ultracentrifugation steps, dialyzed, and titrated (*24*). Usually, titers of approximately 5×10^{10} pfu/mL were obtained. The viruses expressing WT apoE2 and apoE2−202 forms are designated AdGFP-E2 and AdGFP-E2−202, respectively.

Cell Culture Studies. Human HTB13 cells (SW1783, human astrocytoma), grown to confluence in medium containing 10% fetal calf serum (FCS), were infected with AdGFP-E2 or AdGFP-E2−202, at a multiplicity of infection (moi) of 20. Twenty-four hours postinfection, cells were washed twice with phosphate-buffered saline (PBS) and preincubated in serum-free medium for 2 h. Following an additional wash with PBS, fresh serum-free medium was added. After incubation for 24 h, medium was collected and analyzed by SDS−PAGE for apoE expression.

Isolation of VLDL from the Plasma of ApoE and LDL Receptor Double-Deficient ($E^{-/-} \times LDLr^{-/-}$) Mice by Density Gradient Ultracentrifugation and Enrichment of VLDL Particles with ApoE. VLDL was isolated from 1 mL of serum sample obtained from $E^{-/-} \times LDLr^{-/-}$ mice by density gradient ultracentrifugation as described previously (*24*). Two hundred fifty microliters of VLDL was mixed with 750 μL of DMEM containing 15 μg of pure apoE2 and apoE4−202 produced by HTB-13 cells, which were infected with AdGFP-E4 and AdGFP-E4−202, respectively, and the mixtures were brought to a final volume of 1 mL with saline. Mixtures were incubated on a shaker at 37 °C for 30 min and then subjected to density gradient centrifugation to separate free apoE from the VLDL-bound apoE, as described in the VLDL purification step above. Then, the apoE-enriched VLDL and free apoE fractions were isolated and analyzed for apoE concentration by SDS−PAGE and immunoblotting.

Animal Studies, RNA, and Protein Analyses. Female apoE-deficient mice that were 4−6 weeks old were used in these studies. Groups of 8−10 female mice were injected intravenously through the tail vein with doses ranging from 5×10^8 to 1×10^{10} pfu. Blood was obtained from the tail vein after a 4 h fast preceding adenoviral injection and 0, 3, 4, and 5 days after injection. Aliquots of plasma were stored at 4 and −20 °C. Three or more animals from each group were sacrificed on each of the indicated days, and the mRNA levels in the mouse liver were analyzed by Northern blotting, as described previously (*29*−*31*). The ability of apoE2 and apoE2−202 to associate with VLDL obtained from double-deficient mice ($E^{-/-} \times LDLr^{-/-}$) following density gradient ultracentrifugation was performed as described previously (*23*, *24*).

FPLC Analysis and Determination of Lipid Content. For FPLC analysis of serum samples, 12 μL of serum was diluted 1:5 with PBS, loaded onto a Superose 6 column in a SMART micro FPLC system (Pharmacia), and eluted with PBS. A total of 25 fractions with a volume of 50 μL each were collected for further analysis. Triglyceride and cholesterol levels were determined using the GPO-Trinder Kit (Sigma) and CHOL-MPR3 kit (Boehringer-Mannheim), according to the manufacturers' instructions. The triglyceride and cholesterol concentrations of the serum and the FPL fractions were determined spectrophotometrically at 540 and 492 nm, respectively, as described previously (*22*−*24*).

ApoE Production and Purification, and Preparation and Characterization of Discoidal Reconstituted HDL Particles Containing ApoE (POPC−*ApoE).* To generate media for isolation of apoE2, apoE4, and apoE2−202 forms, human astrocytoma HTB-13 cells which do not express apoE were infected with the recombinant adenoviruses expressing these apoE forms and the serum-free medium was collected and

purified by dextran sulfate column chromatography (*27*). POPC−apoE particles were prepared from a POPC:cholesterol:apoE:sodium cholate molar ratio of 100:10:1:100, employing the sodium cholate dialysis method with only minor modifications (*28*). The particles formed were analyzed by EM and protein cross-linking (*27*). ApoE was labeled with ^{125}I using Iodo-Beads (*29*) iodination reagent and Na^{125}I (New England Nuclear). Each reaction used 1 mCi of ^{125}I and three beads, and 1 mg of POPC−apoE particles.

Receptor Binding Assay. ldlA-7 is an LDL receptor-deficient Chinese hamster ovary (CHO) cell mutant (*30*, *31*). The ldlA[LDLr] cells (TR-715-19 cells), a generous gift of J. Goldstein, are ldlA-7 cells stably transfected with the expression plasmid (*32*). Both cell lines were maintained in a monolayer culture in Ham's F12 medium containing 5% fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM glutamine. All incubations with cells were performed at 4 °C in a humidified 5% CO_2/95% air incubator.

LDL receptor binding at 4 °C was assessed by measuring the extent of cell association of radiolabeled ligands. Briefly, on day 0, cells (both ldlA-7 and ldlA[LDLr]) were plated at concentrations of 4.5−5 × 10^4 cells/well in 24-well dishes in complete F12 medium. On day 2, the monolayers were washed twice with Ham's F12 medium and then re-fed with 0.4−0.5 mL of medium [Ham's F-12 containing 0.5% (w/v) FAF-BSA, 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM glutamine] with the indicated radiolabeled [^{125}I]POPC−apoE ligands. Eight different concentrations, ranging from 0.5 to 100 μg/mL, were used, and the experiments were performed in duplicate. After a 1.5 h incubation at 4 °C, the cells were washed twice at 4 °C with buffer B [50 mM Tris-HCl (pH 7.4) and 0.15 M NaCl] containing 2 mg/mL FAF-BSA, followed by one rapid wash with buffer B alone. The cells were then solubilized with 0.1 N NaOH (300 μL each well). Aliquots of 200 μL were used for radioactivity determinations, and aliquots of 25 μL were used for determination of the protein concentration using the BCA assay (*33*).

The level of specific binding was obtained by subtracting the level of binding of the untransfected control cells (ldlA-7) from the level of binding of the receptor-expressing cell lines ldlA[LDLr] (31). Binding parameters K_d and B_{max} were determined on the basis of the specific binding curve using the Prism program (GraphPad Software, Inc.). The specific binding (cell association) values of the saturation curves are expressed in nanograms of apoE in the complex associated with the cells per milligram of total cell protein.

Rate of VLDL Triglyceride Production in C57BL/6 Mice Infected with Different ApoE Forms. VLDL triglyceride secretion was assessed following infection of C57BL/6 mice with 2 × 10^9 pfu of adenoviruses expressing apoE2 or apoE2−202 or the control AdGFP viruses, or a mixture of apoE2 and apoE2−202 adenoviruses. Five days postinfection, mice were fasted for 4 h and then injected with Triton WR1339 at a dose of 500 mg/kg of body weight, using a 15% solution (w/v) in 0.9% NaCl [Triton WR1339 has been shown to completely inhibit VLDL catabolism (*34*)]. Serum samples were isolated 20, 40, 60, and 90 min after injection with Triton WR1339. Serum triglyceride concentrations were measured, and the rate of VLDL triglyceride secretion, expressed in milligrams per deciliter per minute, was determined as described previously (*24*). The means ± the standard deviations of three to four experiments are presented in the form of a bar graph.

RESULTS

Truncated Forms of ApoE2 Have a Dominant Effect in the Correction of Hyperlipidemia in ApoE-Deficient and C57BL/6 Mice. Our previous studies have shown that overexpression of several truncated apoE4 forms lacking different portions of the carboxy-terminal region between residues 185 and 299 can clear cholesterol from the plasma of apoE-deficient mice, whereas overexpression of full-length apoE forms, irrespective of the apoE phenotype, did not clear cholesterol and induced hypertriglyceridemia.

ApoE2 represents an interesting case because it is generally believed that the R158 for C substitution reduces the affinity of apoE for the LDL receptor and results in type III hyperlipoproteinemia (*20*).

To assess the effects of apoE2 and apoE2−202 on hyperlipidemia *in vivo*, apoE-deficient mice (E$^{-/-}$) were infected with the recombinant adenoviruses expressing apoE2 or apoE2−202. This analysis showed that the infection of mice with 2 × 10^9 pfu of the apoE2 adenovirus exacerbated the plasma cholesterol levels of the E$^{-/-}$ mice 4 days postinfection (Figure 1A). In addition, the infection induced severe hypertriglyceridemia as was observed previously for other apoE phenotypes (*22*−*24*) (Figure 1B).

In parallel experiments, we infected C57BL/6 mice with 2 × 10^9 pfu of adenoviruses expressing apoE2, apoE2−202, or a mixture of the full-length apoE2 and the truncated apoE2−202 forms. This analysis showed that overexpression of apoE2 was sufficient to induce combined hyperlipidemia (high cholesterol and triglyceride levels) in normal C57BL/6 mice (Figure 1A,B), consistent with our previous findings (*23*). Similar results were obtained by overexpression of apoE4 (data not shown). The control virus AdGFP did not appear to have a significant effect on the cholesterol and triglyceride levels as compared to the levels of noninfected C57BL/6 mice, ruling out the possibility of nonspecific effects of the infection process (data not shown). The most interesting finding, however, was that overexpression of apoE2−202 or an equal dose of the apoE2 and apoE2−202 mixture had no effect on plasma lipid levels of the C57BL/6 mice (Figure 1A,B), indicating that truncated apoE forms have a dominant effect in the clearance of lipoprotein remnants.

Determination of the Level of ApoE Expression in Mice and Cells and Control Experiments. To assess the expression of apoE in mice infected with the adenoviruses expressing apoE2 or apoE2−202, total RNA was isolated from the mouse livers 5 days postinfection and analyzed for apoE and GAPDH mRNA levels by Northern blot analysis. This analysis showed that the hepatic mRNA levels of E$^{-/-}$ mice infected with the apoE2- or apoE2−202-expressing adenoviruses were similar (Figure 2A). However, apoE2−202 efficiently clears the cholesterol from the plasma of E$^{-/-}$ mice without causing hypertriglyceridemia, whereas the full-length apoE2 does not clear cholesterol from the plasma of E$^{-/-}$ mice and causes hypertriglyceridemia in E$^{-/-}$ mice (Figure 2B,C). Similar observations were made in C57BL/6

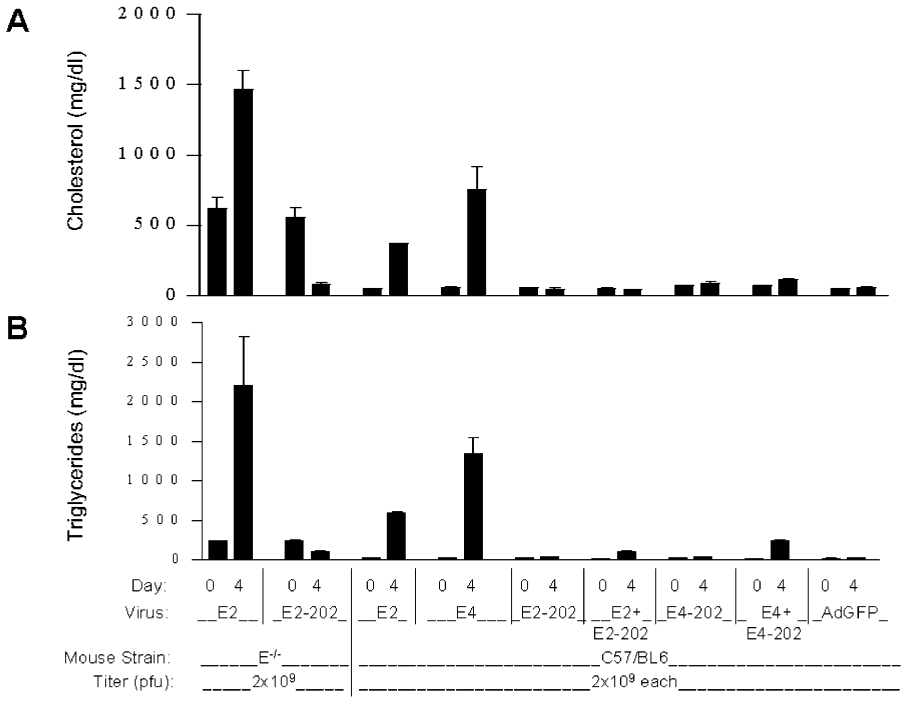

FIGURE 1: Cholesterol and triglyceride levels of $E^{-/-}$ or normal C57BL/6 mice infected with the control adenovirus AdGFP, or recombinant adenoviruses expressing apoE2, apoE2−202, or a mixture of apoE2 and apoE2−202. Three mice were infected with the indicated doses of a specific recombinant virus, and serum samples were isolated and analyzed for cholesterol (A) and triglyceride levels (B) on the indicated days after infection as described in Experimental Procedures.

mice infected with adenoviruses expressing apoE2, apoE2−202, or a mixture of the two. In these experiments, only the mice expressing the full-length apoE2 forms are characterized by combined hyperlipidemia, despite the fact that the hepatic mRNA levels of the corresponding full-length and truncated apoE2−202 are comparable (Figure 2A−C). Similar results were obtained for C57BL/6 mice infected with adenovirus apoE4, adenovirus apoE4−202, or a mixture of the two (data not shown). The findings indicate that the truncated apoE forms, independent of the apoE phenotype, have a dominant effect in the clearance of the lipoprotein remnants when they are coexpressed along with full-length apoE forms.

In other control experiments, analysis of the culture medium by SDS−PAGE and sandwich ELISA showed that following adenovirus infection, in HTB-13 cells both apoE2 and apoE2−202 are secreted efficiently at comparable levels in the range of 150−200 μg of apoE/mL (Figure 3A). The ability of lipid-free apoE2 and apoE2−202 to associate with VLDL isolated from the plasma of $E^{-/-} \times LDLr^{-/-}$ double-deficient mice was also assessed by ultracentrifugation of the mixture (VLDL+apoE). The amounts of free and lipoprotein-associated apoE were assessed by fractionation via SDS−PAGE followed by Western blot analysis for apoE. It was found that both the full-length apoE2 and the truncated apoE2−202 associate with particles with densities in the VLDL to HDL region (Figure 3B,C). The ability of apoE to associate with lipoproteins is essential for remnant clearance.

Cholesterol and Triglyceride FPLC Profiles of Plasma Isolated from Mice Infected with Adenoviruses Expressing the Full-Length or Truncated ApoE2 Forms. FPLC analysis of plasma from adenovirus-infected $E^{-/-}$ mice or C57BL/6 mice expressing apoE2, 4 days postinfection, showed cholesterol levels were high and were predominantly distributed in the VLDL region (Figure 4A,B, top panel). In $apoE^{-/-}$ and C57BL/6 mice expressing apoE2−202, cholesterol was distributed in the VLDL and HDL and the ratio of VLDL cholesterol to HDL cholesterol was approximately 1:1 (Figure 4A,B, bottom panel). In mice expressing both apoE2 and apoE2−202, approximately 90% of the cholesterol was distributed in the HDL and 10% in the VLDL (Figure 4B, bottom panel). In $E^{-/-}$ or C57BL/6 mice infected with an E2-expressing adenovirus, as expected, triglyceride levels were high in the VLDL region and barely detectable in the rest of the lipoprotein fractions (Figure 5A,B, top panel). In mice expressing apoE2−202 or both apoE2 and apoE2−202, triglyceride levels were not detectable in the $E^{-/-}$ mice, whereas in the C57BL/6 mice, very low levels of triglycerides were found mostly in the VLDL region (Figure 5A,B, bottom panels).

The steady-state level of apoE2−202 is very low in the range of 2−3 mg/dL (consistent with the efficient clearance of apoE2−202-containing lipoproteins), whereas the steady-state levels of the full-length apoE2 were determined to be between 60 and 80 mg/dL. ApoE2−202 is distributed all across the lipoprotein fractions. ApoE2 is distributed in the VLDL and HDL regions (data not shown). This pattern of distribution is similar to that described previously for apoE4 and apoE4−202 (*23*).

ApoE2 Significantly Increases the Rate of Hepatic VLDL Triglyceride Secretion in C57BL/6 Mice As Compared to That with the Truncated ApoE2−202 Form or a Mixture of ApoE2 and ApoE2−202. The rate of VLDL triglyceride secretion in the plasma was determined following injection of Triton WR1339 5 days after the infection with the recombinant adenoviruses. It was found that the rate of triglyceride secretion was slightly decreased in mice infected with adenovirus expressing E2−202 and increased 4.1-fold in mice infected with adenovirus expressing apoE2, and

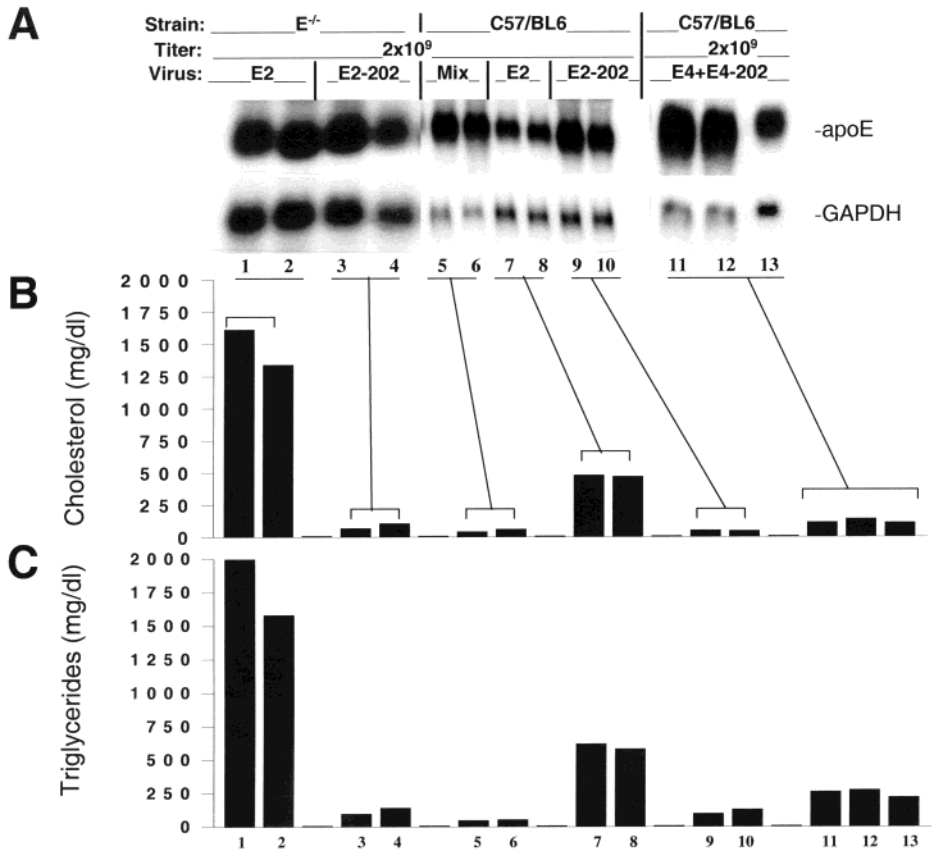

FIGURE 2: Analysis of total RNA isolated from livers of C57BL/6 mice infected 5 days postinfection. The samples were analyzed by Northern blotting for the expression of apoE and GAPDH mRNA. (A) Representative autoradiograms of Northern blot analysis of total RNA isolated from livers of individual mice infected with the indicated dose of the recombinant adenoviruses expressing apoE2, apoE2−202, or a mixture of both viruses. (B) Cholesterol levels of the individual mice expressed in milligrams per deciliter. (C) Triglyceride levels of the individual mice expressed in milligrams per deciliter.

approximately 3.0-fold in mice infected with a mixture of adenoviruses expressing apoE2 and apoE2−202 as compared to mice infected with the control AdGFP adenovirus (Figure 6). The findings with full-length apoE2 in C57BL/6 mice are similar to the findings with the full-length apoE forms in $E^{-/-}$ mice (*22*, *24*). Thus, the effect of apoE on VLDL triglyceride secretion appears to be independent of the apoE phenotype and of mouse strain. The finding that increased VLDL triglyceride secretion occurs with the mixture of full-length and truncated apoE indicates a dominant effect of the carboxy-terminal segment of apoE in the observed increase in the rate of VLDL secretion. These observations suggest that the presence of the carboxy-terminal region of residues 203−299 in the full-length apoE influences the rate of VLDL triglyceride secretion, and contributes to apoE-induced hypertriglyceridemia.

Coexpression of Full-Length ApoE2 and Lipoprotein Lipase Corrects the ApoE2-Induced Hypertriglyceridemia but Retains ApoE Induced Hypercholesterolemia in C57BL/6 Mice. The induction of combined hyperlipidemia in C57BL/6 mice could be the result of the insufficiency of the circulating lipoprotein lipase activity, apoCII, or both. To test these possibilities, C57BL/6 mice were infected with either 1×10^9 pfu of the adenovirus-expressing E2 alone or a mixture of 1×10^9 pfu of apoE2-expressing adenovirus and 10^9 pfu of adenovirus expressing the WT or a mutant form of human lipoprotein lipase. This analysis showed that coinfection with the adenovirus expressing the WT lipoprotein lipase (Ad-LpL) corrected the apoE-induced hypertriglyceridemia in the C57BL/6 mice (Figure 7A). The findings indicate that under conditions of apoE overexpression, the endogenous lipoprotein lipase activity may be rate-limiting for the lipolysis and/or clearance of VLDL.

To assess the independent contribution of lipoprotein lipase in remnant clearance, we used an inactive form of lipoprotein lipase having S132 for T substitution (*35*). Coinfection of mice with adenoviruses expressing apoE2 and the inactive form of lipoprotein lipase was associated with a less severe form of hypertriglyceridemia. The triglyceride levels in these mice were 60% of the levels in mice infected with apoE2 alone (Figure 7A). Thus, it appears that the inactive form of lipoprotein lipase may contribute partially to the clearance of triglyceride-rich VLDL particles.

Analysis of the plasma cholesterol in mice infected with adenoviruses expressing different combinations of apoE2 and lipoprotein lipase showed that under conditions of apoE2 overexpression, neither the active nor the inactive form of lipoprotein lipase was able to reverse the apoE2-induced hypercholesterolemia (Figure 7B). Comparative analysis of the plasma FPLC profiles of the infected mice showed that in the presence of inactive LPL, triglycerides were distributed in VLDL and their profile resembles the FPLC triglyceride profile of mice infected with apoE2 alone. However, the FPLC cholesterol profile of mice infected with apoE2 and the active form of LPL was distributed in the VLDL, IDL, and HDL region, whereas in mice expressing apoE2 and the inactive form of LPL, cholesterol was mainly distributed in the VLDL region (Figure 7C). The findings suggest that

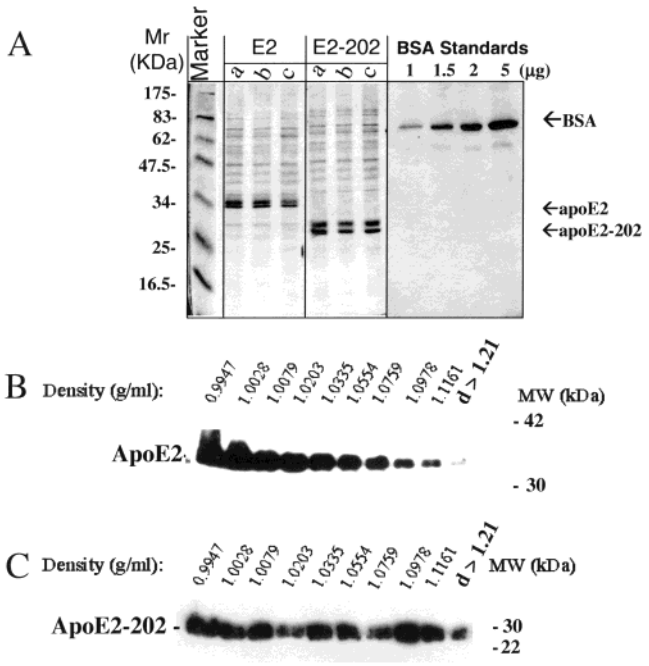

FIGURE 3: Expression of apoE2 and apoE2−202 in cultures of HTB-13 cells infected with AdGFP-E2 and AdGFP-E2−202, respectively. (A) SDS−PAGE analysis of the culture medium of HTB-13 cells infected with adenoviruses expressing apoE2 and apoE2−202. Ten microliters of culture medium was analyzed. "Marker" indicates protein markers with different M_r's, as indicated in the figure. (B and C) Western blot analysis of apoE2 and apoE2−202 fractions bound to VLDL particles from apoE and LDLR double-deficient mice. Approximately 15 μg of apoE2 and apoE2−202 secreted by adenovirus-infected HTB-13 cells was mixed with VLDL isolated from plasma of apoE and LDLR double-deficient mice, and incubated at 37 °C for 30 min. Then the VLDL-associated apoE was separated from free apoE by density gradient ultracentrifugation and analyzed by Western blot analysis as described in Experimental Procedures.

under conditions of apoE overexpression, lipolysis may not be sufficient to promote lipoprotein clearance.

Deletion of Carboxy-Terminal Residues 203−299 of ApoE2 Increases the Affinity of POPC−ApoE2 Particles for the LDL Receptor. To explain the dominant effect of the truncated apoE2−202 form in the clearance of lipoprotein remnants *in vivo*, we examined the affinity of different POPC−apoE particles for the LDL receptor. The POPC−apoE particles were generated by the sodium cholate dialysis method and analyzed by EM as described in Experimental Procedures. Cross-linking of the particles with sodium suberimidate showed the presence of two apoE2 or apoE2−202 molecules per particle. The average size of POPC particles, determined from the EM pictures, was 174 ± 49 Å (*27*). It is known that different cells contain several apoE-recognizing receptors (*36*). To establish the specific binding of POPC−apoE particles to the LDL receptor, it was necessary to subtract the background level of binding of apoE to all these receptors. For this purpose, binding studies were performed in ldlA-7 cells stably expressing the LDL receptor (LDLr cells), and in untransfected ldlA-7 cells. The specific binding curve and the binding parameters K_d and B_{max} were then determined by subtracting the values for binding to the ldlA cell for every experimental point from the corresponding values for binding to ldlA[LDLr] cells (Figure 8). Binding parameters K_d and B_{max} were determined on the basis of the specific binding curve using the Prism program (GraphPad Software Inc.). This analysis showed that the K_d values of POPC−apoE2, −apoE2−202, and −apoE4 particles were 29.0 ± 3.1, 9 ± 2.0, and 7.0 ± 0.8 μg of protein/mL, respectively. The findings confirmed the previously reported reduced affinity of phospholipid-bound apoE2 for the LDL receptor compared to that of the apoE4 isoform (*20*), and indicated that removal of carboxy-terminal residues 203−

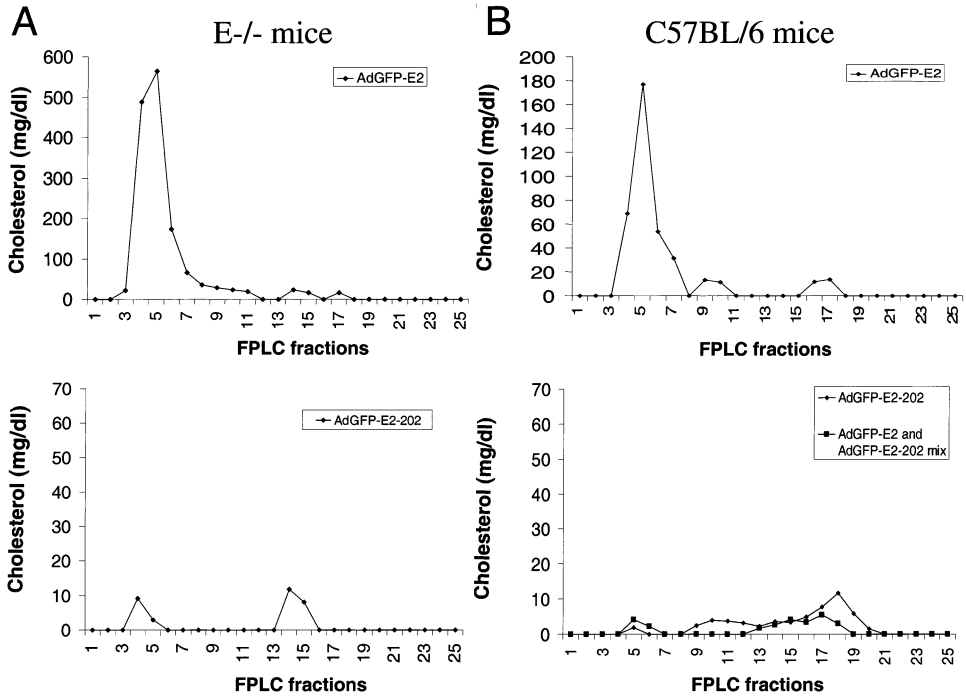

FIGURE 4: FPLC profiles of cholesterol in $E^{-/-}$ (A) and C57BL/6 (B) mice. Serum samples obtained from mice infected with 2×10^9 pfu of the recombinant adenoviruses expressing AdGFP-E2 or AdGFP-E2−202 or a mixture of AdGFP-E2 and AdGFP-E2−202, on day 5 post-infection. The samples were fractionated by FPLC, and then the cholesterol levels of each FPLC fraction were determined as described in Experimental Procedures.

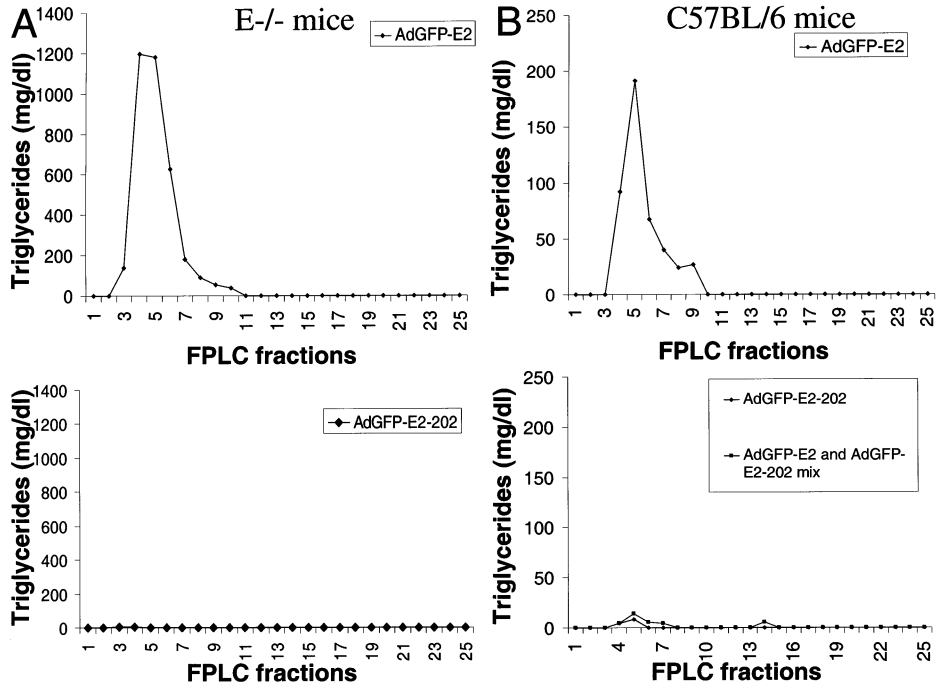

FIGURE 5: FPLC profiles of triglycerides in $E^{-/-}$ (A) and C57BL/6 (B) mice. Serum samples obtained from mice infected with 2×10^9 pfu of the recombinant adenoviruses expressing AdGFP-E2, AdGFP-E2−202, or a mixture of AdGFP-E2 and AdGFP-E2−202 on day 5 post-infection. The samples were fractionated by FPLC, and then the triglyceride levels of each FPLC fraction were determined as described in Experimental Procedures.

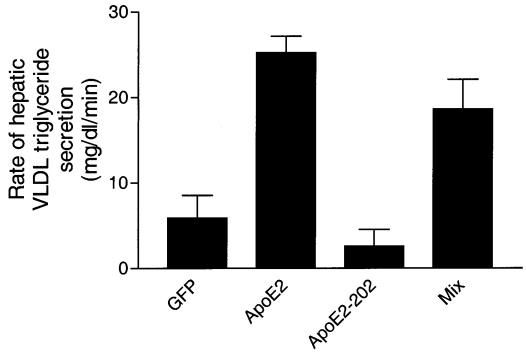

FIGURE 6: Hepatic VLDL triglyceride production analysis in mice infected with AdGFP, AdGFP-E2, AdGFP-E2−202, or a mixture of AdGFP-E2 and AdGFP-E2−202. Triton WR1339 (500 mg/kg of body weight) was injected into three fasting mice. Serum samples were collected 20, 40, 60, and 90 min after the detergent had been injected. Control serum samples were isolated 1 min immediately after the injection with the detergent. Serum triglyceride levels were determined, and a linear graph of serum triglyceride concentration vs time was generated. The rate of VLDL triglyceride secretion expressed in milligrams per deciliter per minute was calculated from the slope of the linear graph for each individual mouse. The means ± standard deviations of the individual rates of VLDL triglyceride production are presented in the form of bar graphs.

299 of apoE2 increases the affinity of POPC−apoE2 particles for the LDL receptor.

Truncated ApoE Forms Cannot Clear Cholesterol in Double-Deficient $E^{-/-} \times LDLr^{-/-}$ Mice, Suggesting that the LDL Receptor Is the Predominant Receptor for Remnant Clearance. To assess the contribution of receptors other than the LDL receptor in the clearance of plasma lipoproteins, we attempted to correct the high cholesterol content of $E^{-/-} \times LDLr^{-/-}$ mice by infecting these mice with 2×10^9 pfu of adenovirus expressing full-length apoE2 or apoE4 and truncated apoE2−202 or apoE4−202. Analysis of the plasma cholesterol and triglyceride levels showed that in mice infected with the apoE2- or apoE4-expressing virus, there was a dramatic 2.5-fold increase in the level of plasma cholesterol that was also associated with severe hypertriglyceridemia (Figure 8A,B). The triglyceride levels of the apoE2- and apoE4-expressing mice were between 1500 and 2000 mg/dL 5 days postinfection. In mice infected with the apoE2−202- or apoE4−202-expressing virus, cholesterol and triglyceride levels were not altered significantly compared to those in $E^{-/-}$ mice. This pattern of lipoprotein clearance in the $E^{-/-} \times LDLr^{-/-}$ double-deficient mice differs from the pattern of remnant clearance by full-length and truncated apoE forms observed in $E^{-/-}$ mice. The results in panels A and B of Figure 9 provide two novel findings about the role of apoE in hypertriglyceridemia and remnant clearance. The first is that remnant clearance by the truncated apoE forms requires the presence of the LDL receptor. The second is that although in the absence of the LDL receptor cholesterol is not cleared by the truncated apoE forms, overexpression of the truncated apoE forms does not induce hypertriglyceridemia.

DISCUSSION

Background. Familial type III hyperlipoproteinemia, also called dysbetalipoproteinemia, broad b, or floating b disease, is characterized by premature atherosclerosis, xanthomas, elevated plasma cholesterol and triglyceride levels, cholesterol-enriched b VLDL and IDL particles, increased plasma apoE levels, and premature coronary and peripheral atherosclerosis (*1*, *37*). The most reliable criterion used in the past for the diagnosis of this disease was an increase in the ratio of VLDL cholesterol to total triglycerides and a plasma triglyceride concentration between 150 and 1000 mg/dL (*1*, *2*). Initial studies of the phenotypes of the patients who were diagnosed with the above criteria to have type III hyperlipoproteinemia showed that 31 of the 34 patients had the E2/2 phenotype

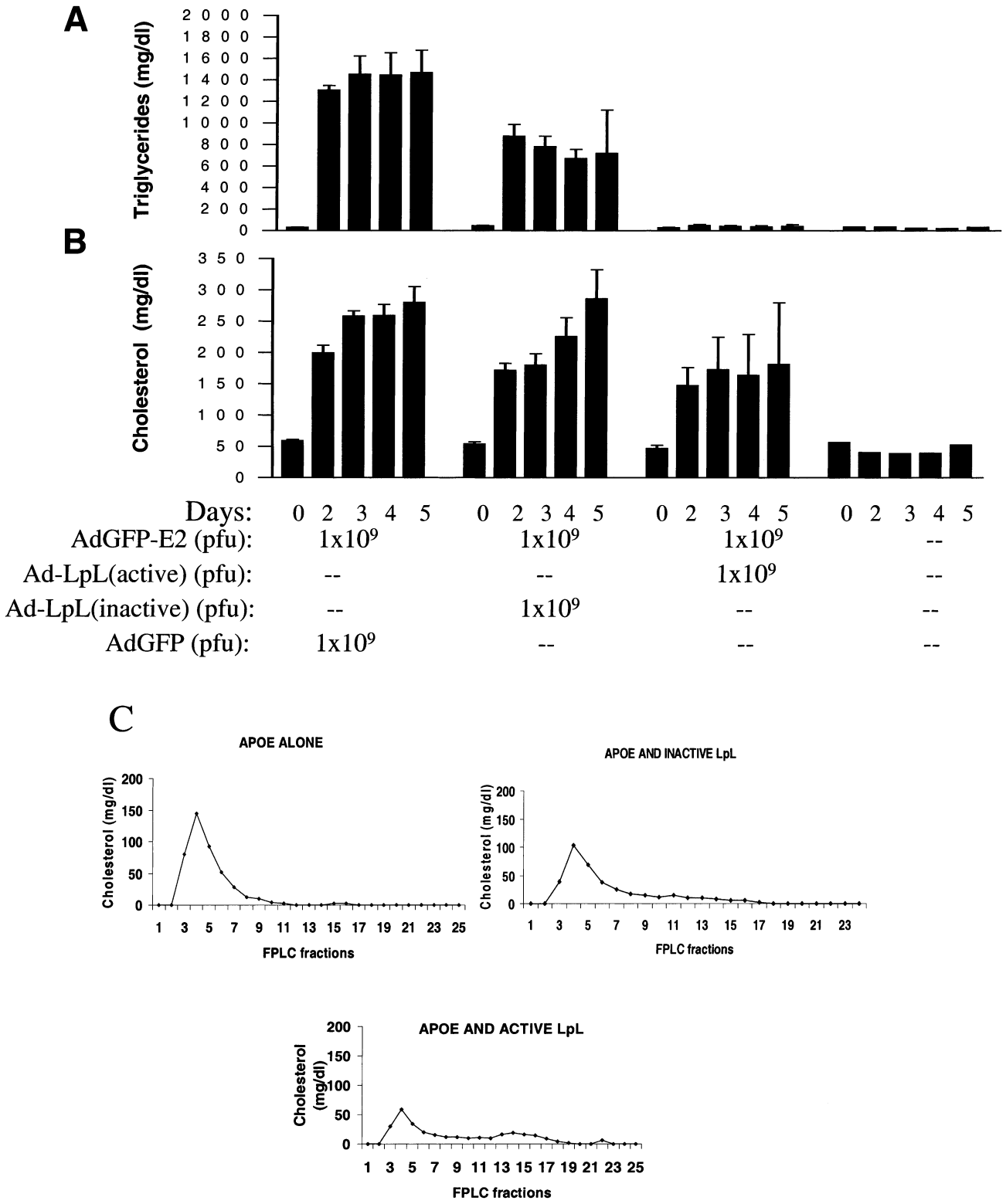

FIGURE 7: Plasma lipids and FPLC cholesterol profiles of mice infected with apoE2 or a combination of apoE2 with an active or inactive form of lipoprotein lipase. (A and B) Triglyceride and cholesterol levels of C57BL/6 mice infected with a recombinant adenovirus expressing either full-length apoE2 alone or a mixture of apoE2 and an active or an inactive form of human lipoprotein lipase or noninfected mice. Mice were infected with the indicated doses of the recombinant adenoviruses, and serum samples were isolated and analyzed for triglyceride and cholesterol levels 0, 2, 3, 4, or 5 days postinfection, as described in Experimental Procedures. (C) Representative FPLC cholesterol profile of plasma from mice infected with a mixture of E2 alone or a mixture of apoE2 and an active or inactive form of LpL.

(*38*, *39*). Thus, the apoE phenotype E2/2 could serve as a molecular marker in 91% of the patients with type III hyperlipoproteinemia. It has been proposed that residue 158 of apoE may not be involved directly in receptor binding but may indirectly affect the conformation of the receptor binding domain of apoE located in the vicinity of residues 140−150 (*40*). Patients with mutations in this region have a dominant form of type III hypertriglyceridemia (*41*), whereas apoE2, which has 158C, is associated with a recessive form of type III hyperlipoproteinemia (*38*, *39*, *41*). Previous

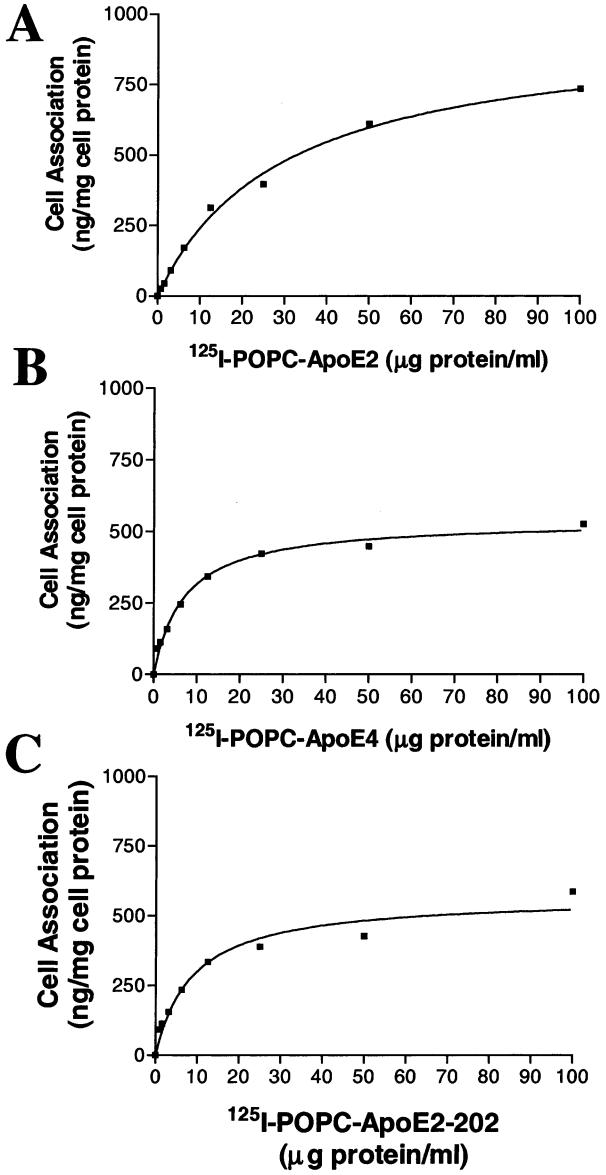

FIGURE 8: Representative curves of the concentration-dependent binding of [^{125}I]POPC−apoE complexes to confluent monolayers of ldlA CHO cells expressing the LDL receptor and control ldlA CHO cells. (A and B) ldlA-7 cells and ldlA[LDLr] CHO cells in the microtiter wells were washed and incubated with various concentrations of [^{125}I]POPC−apoE particles. The levels of total binding to the permanent ldlA[LDLr] cells and total binding to the untransfected ldlA-7 cells were obtained experimentally. The level of specific binding was determined by subtracting the levels for binding of the ldlA-7 cells from the corresponding values for binding to the permanent cell line expressing the LDL receptor. Two independent experiments were preformed in duplicate for each apoE form. The apoE forms that were used were (A) apoE2, (B) apoE4, and (C) apoE2−202. The average K_d values for apoE2, apoE4, and apoE2−202 are 29 ± 3.1, 7 ± 0.8, and 9 ± 2 μg of protein/mL, respectively. The average B_{max} values for apoE2, apoE4, and apoE2−202 are 750 ± 75, 523 ± 38, and 548 ± 42 ng/mg of cell protein, respectively.

binding and catabolic studies (*18*−*20*, *42*, *43*) are consistent with the observed accumulation of remnant lipoproteins in the plasma of patients with type III HLP who have high levels of cholesteryl esters and apoE (*44*−*48*). These apoE-rich lipoprotein remnants are apparently the result of slow clearance *in vivo* of apoE-containing lipoproteins due to the structural defects in apoE.

Numerous studies suggest that the E2/2 phenotype alone which results from 158R for C substitution is not sufficient to cause type III HLP and that other genetic factors may be required for the phenotypic expression of the disease (*21*, *49*−*51*). In some cases, type III HLP is expressed in families with a tendency toward hypertriglyceridemia based on environmental or other genetic factors (*21*). The concept that other genetic and/or environmental factors may be required for the expression of type III HLP has received direct biochemical support from studies by Rall et al. (*41*). These investigators have shown that apoE from subjects with the apoE2/2 phenotype who are normolipidemic or hypolipidemic behaves similarly in competition experiments, like the apoE2 obtained from patients with type III hyperlipoproteinemia (*41*).

Truncated ApoE Forms Have a Dominant Effect on Remnant Clearance. This study showed that overexpression of full-length apoE2 in $E^{-/-}$ mice is associated with high cholesterol and triglyceride levels, whereas overexpression of the truncated apoE2−202 normalizes cholesterol levels of $E^{-/-}$ mice and does not trigger hypertriglyceridemia. Thus, full-length apoE2 behaves *in vivo* like the apoE3 and apoE4 isoforms (*22*, *23*) except that it exacerbated the high cholesterol levels of $E^{-/-}$ mice. Unexpectedly, however, the truncated apoE2−202 form behaves *in vivo* like the truncated apoE4−202 form (*24*) despite the fact that apoE2−202 retains the R158 for C substitution.

The hypertriglyceridemia induced in $E^{-/-}$ mice by overexpression of the human apoE2 could be the consequence of the underlying hypercholesterolemia. Alternatively, full-length apoE2 could by itself elicit high plasma lipid levels. To differentiate between these two possibilities, we infected C57BL/6 mice with 2×10^9 pfu of adenoviruses expressing wild-type apoE2, and the truncated apoE2−202. Consistent with previous findings with apoE3 and apoE4 (*23*), this analysis showed that only the overexpression of full-length apoE2 in the liver induces combined hyperlipidemia in C57BL/6 mice, characterized by high plasma cholesterol and triglyceride levels, whereas overexpression of apoE2−202 has no effect on plasma cholesterol and triglyceride levels (Figure 1). The most important observation, however, was that the hyperlipidemic effect of apoE2 could be reversed by coexpression of full-length apoE2 and the truncated apoE2−202 form. Similar results were obtained by overexpression of a mixture of apoE4−202- and apoE4-expressing adenoviruses (data not shown). These findings establish that the truncated apoE2 forms have a dominant effect in the clearance of lipoprotein remnants.

Coexpression of Full-Length ApoE2 and Truncated ApoE2−*202 Increases VLDL Triglyceride Secretion.* Previous studies (*22*, *24*) showed that overexpression of full-length apoE4 increases hepatic VLDL triglyceride secretion, whereas overexpression of truncated apoE4−202, −185, and −202 has no effect on VLDL triglyceride secretion. In this study, we examined the effect of full-length and truncated apoE forms on VLDL triglyceride secretion in C57BL/6 mice. Consistent with the previous findings, only the full-length form apoE2 but not the truncated apoE2−202 increased 4.1-fold the rate of VLDL triglyceride secretion in C57BL/6 mice. However, a 3.0-fold increase in the rate of VLDL triglyceride secretion was also observed when the mice were infected with a mixture of adenoviruses expressing apoE2

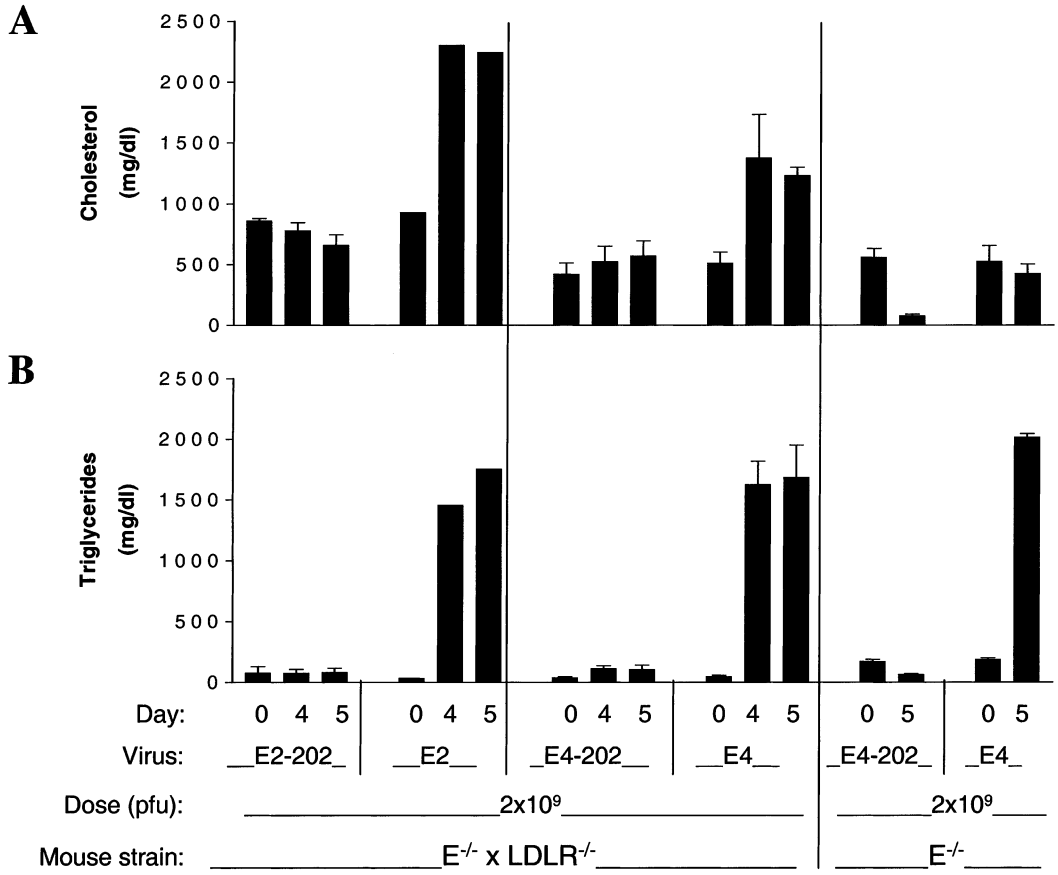

FIGURE 9: Cholesterol and triglyceride levels of $E^{-/-} \times LDLr^{-/-}$ double-deficient mice infected with recombinant adenoviruses expressing the full-length apoE2, apoE4, or the truncated apoE2−202, apoE4−202. Three mice were infected with the indicated doses of specific recombinant viruses, and serum samples were isolated and analyzed for cholesterol and triglyceride levels on the indicated days after infection as described in Experimental Procedures.

and apoE2−202. Similar results were obtained by infection of mice with a mixture of adenoviruses expressing apoE4 and apoE4−202. The data suggest that full-length apoE has a dominant effect in VLDL triglyceride secretion, and the simultaneous expression of truncated apoE cannot reverse this effect. It has been suggested that apoE may participate in the last step of intracellular assembly of VLDL which involves the addition of triglycerides to the nascent VLDL (*52*). It is possible that participation of apoE in VLDL assembly may require the carboxy-terminal domain of the protein.

Mechanism of ApoE-Induced Hypertriglyceridemia. We have shown previously that the VLDL of mouse infected with full-length apoE4 is greatly enriched with apoE4 (*23*). Thus, it appears that the lipoproteins secreted when apoE is overexpressed acquire apoE intracellularly or extracellularly and become resistant to further catabolism. This implies that the triglycerides of these particles cannot be hydrolyzed, and also cannot be recognized by lipoprotein receptors. It is possible that the full-length apoE assumes a configuration on the lipoprotein particle that masks its receptor binding domain. It has recently been proposed on the basis of physicochemical studies that at high apoE concentrations, the carboxy-terminal segment of apoE may displace the amino-terminal helices and expose them to the solvent (Figure 10A) (*53*). Since apoE in solution is not a ligand for lipoprotein receptors, particles containing a full-length apoE with the amino-terminal helices exposed to the solvent would not be cleared by cell receptors. Such an interpretation, however, cannot explain the dominant effect of the truncated apoE2−202 form in the clearance of lipoprotein particles

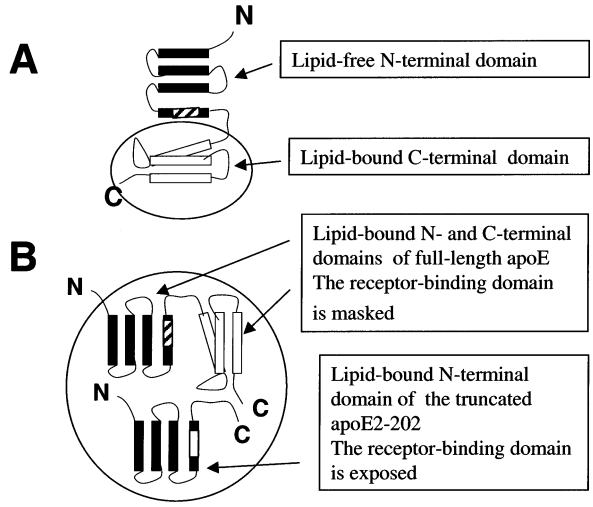

FIGURE 10: Proposed models of association of full-length apoE with lipoproteins. (A) The model proposed by Saito et al. (*53*) assumes that in the presence of an excess of full-length apoE, only the C-terminal helices are bound to lipoproteins whereas the amino-terminal helices are not bound to lipoproteins and are exposed to the solvent. (B) Our work suggests that both the amino- and carboxy-terminal domains of full-length apoE as well as the carboxy-terminal domain of truncated apoE are bound to lipoproteins. However, the receptor binding domain of full-length apoE is masked, whereas the receptor binding domain of truncated apoE is accessible to lipoprotein receptors.

that are formed by overexpression of both apoE2 and apoE2−202. The *in vivo* studies that we present suggest that the truncated apoE2−202 form, which contains only the amino-terminal helices of apoE2, must associate with lipoprotein particles which also contain full-length apoE2, and direct the clearance of these particles by cell receptors (Figure 10B). The fact that full-length apoE alone cannot promote remnant clearance suggests that the amino-terminal portion of the full-length apoE bound to lipoprotein particles assumes a configuration that masks the receptor binding domain (Figure 10B).

Other studies have suggested that an excess of secreted apoE may displace partially the lipoprotein lipase and/or apoCII and reduce the rate of lipoprotein triglyceride lipolysis (*54*, *55*). As a result, lipoprotein particles enriched in cholesterol, triglycerides, and apoE accumulate in the plasma of apoE-overexpressing mice. Similar particles have been shown to accumulate in the plasma of patients with type III hyperlipoproteinemia (*1*, *39*). Our study showed that the increase in the plasma levels of the wild-type lipoprotein lipase by infection with recombinant adenoviruses expressing the human lipoprotein lipase corrected completely the apoE-induced hypertriglyceridemia in C57BL/6 mice. This suggests that the activity of lipoprotein lipase rather than apoCII becomes rate-limiting for the clearance of VLDL triglycerides. The increased levels of plasma lipoprotein lipase may promote lipoprotein clearance either by increasing the rate of lipolysis, by direct receptor-mediated clearance mechanisms (*56*, *57*), or by a combination of both processes. The observation that the inactive form of lipoprotein lipase corrects partially the apoE2-induced hypertriglyceridemia indicates that under the experimental conditions that were used, both the improved lipolysis and the receptor-mediated clearance may contribute to the reduction of the plasma triglyceride levels.

The Carboxy-Terminal Region of Residues 203−299 of ApoE2 Influences the Affinity of POPC−ApoE2 Particles for the LDL Receptor. One possible explanation for the efficient clearance of the lipoprotein remnants by the truncated apoE2−202 form in mice which coexpress apoE2 and apoE2−202 could be the increased affinity for the LDL receptor and possibly other lipoprotein receptors. In this study, receptor binding experiments showed that the removal of carboxy-terminal amino acids 203−299 of apoE2 increased the affinity of POPC−apoE2−202 particles for the LDL receptor. The experiments also confirmed the increased affinity of POPC−apoE4 particles as compared to the affinity of POPC−apoE2 particles for the LDL receptor ($K_d = 7 \pm 0.8$ vs 29 ± 3.1 μg of protein/mL) (*20*, *41*).

It is possible that the presumed distortion of the receptor binding domain of apoE2 on the lipoprotein remnants is the result of hypertriglyceridemia which occurs in a subfraction of individuals who have the apoE2/2 phenotype (*21*, *39*). Previous studies established that apoE2 obtained from normolipidemic or hyperlipidemic subjects behaves similarly in receptor binding and competition studies (*41*). However, *in vivo*, the dyslipidemic subjects present the same remnant clearance defect as the mice overexpressing full-length apoE2, apoE3, or apoE4 isoforms (*22*−*24*). Although the conformation of the full-length apoE is expected to be different on the discoidal phospholipid-rich lipoprotein particles as compared to the spherical lipoprotein particles, these observations support strongly the hypothesis that the association of apoE with different lipoprotein particles may alter the conformation of the amino acids in the vicinity of the receptor-binding site. Since the carboxy-terminal segment of residues 203−299 of apoE folds into an independent domain and contributes to the binding of apoE to lipids and lipoproteins, it is possible that binding of apoE2 to triglyceride-rich VLDL may distort or mask the receptor binding site of apoE, thus preventing receptor-mediated clearance of triglyceride-rich VLDL.

The LDL Receptor Is the Predominant Receptor in Remnant Clearance. This study and the previous studies showed that truncated apoE forms mediate the efficient clearance of apoE-containing lipoproteins *in vivo* (*22*−*24*). The efficient apoE-mediated clearance of lipoprotein remnants also results in the concomitant clearance of the apoE molecules, thus resulting in lower steady-state plasma apoE levels (*24*). Work by others showed that injection of two truncated [^{125}I]apoE forms, extending from residue 1 to 191 and from residues 1 to 244 in rabbits, resulted in the fast and very efficient removal of the molecules from plasma (*58*). These observations suggested that truncated apoE forms may direct more efficient lipoprotein clearance via the LDL receptor or other receptors such as LRP. The current study establishes that whereas overexpression of truncated apoE2−202 can normalize the cholesterol and triglyceride levels of the $E^{-/-}$ mice, similar doses of apoE2−202 did not correct the high cholesterol level of the $E^{-/-} \times LDLr^{-/-}$ double-deficient mice. Furthermore, infection of the $E^{-/-} \times LDLr^{-/-}$ double-deficient mice with a dose of 2×10^9 pfu of E2 increased 2.5-fold the cholesterol levels and caused severe hypertriglyceridemia. Similar results were obtained by infections of $E^{-/-} \times LDL^{-/-}$ mice with apoE4−202 and apoE4. In this case, infection with apoE4 caused severe hypertriglyceridemia with triglyceride levels in the range of 2000 mg/dL. The data indicate that under conditions of apoE overexpression, lipoprotein clearance by the truncated apoE forms is mediated mostly by the LDL receptor. In the absence of this receptor, LRP and heparan sulfate proteoglycans are not sufficient to clear the lipoproteins, which accumulate in the plasma of the double-deficient mice. The deficiency of the LDL receptor in these mice exacerbates the hypertriglyceridemic effect caused by overexpression of the full-length apoE, independent of the apoE phenotype. The observation that truncated apoE2−202 clears plasma cholesterol but does not induce hypertriglyceridemia in the double-deficient mice implies that induction of hypertriglyceridemia also requires the presence of the carboxy-terminal domain of apoE.

Overall, the current study establishes the following. (a) The dyslipidemia induced by E2/2 in mice and possibly human patients with the E2/E2 phenotype is not the result of R158 for C substitution but is rather brought about by carboxy-terminal residues 203−299 of apoE2. ApoE forms that lack the carboxy-terminal segment cannot induce hypertriglyceridemia in $E^{-/-}$ or $E^{-/-} \times LDL^{-/-}$ mice. (b) Overexpression of apoE increases the secretion of triglyceride-enriched VLDL particles that are resistant to lipolysis. The increased secretion of VLDL cannot be remedied by overexpression of a mixture of full-length and truncated apoE forms. (c) Overexpression of LpL can completely correct the hypertriglyceridemia but not the hypercholesterolemia

that is induced by apoE overexpression. (d) Truncated apoE forms cannot clear the cholesterol from the plasma of the $E^{-/-} \times LDL^{-/-}$ mice but do not induce hypertriglyceridemia. This suggests that the LDL receptor is the predominant receptor in the clearance of apoE-containing lipoprotein remnants and provides further evidence that induction of hypertriglyceridemia requires the carboxy-terminal domain of apoE. (e) Truncated apoE forms, when they are coexpressed with full-length apoE, have a dominant effect on the clearance of the lipoprotein remnants. This suggests that truncated apoE forms may have therapeutic applications in the correction of remnant removal disorders in humans.

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