# Generation of a Recombinant Apolipoprotein E Variant with Improved Biological Functions

HYDROPHOBIC RESIDUES (LEU-261, TRP-264, PHE-265, LEU-268, VAL-269) OF apoE CAN ACCOUNT FOR THE apoE-INDUCED HYPERTRIGLYCERIDEMIA*

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To identify the residues in the carboxyl-terminal region 260–299 of human apolipoprotein E (apoE) that contribute to hypertriglyceridemia, two sets of conserved, hydrophobic amino acids between residues 261 and 283 were mutated to alanines, and recombinant adenoviruses expressing these apoE mutants were generated. Adenovirus-mediated gene transfer of apoE4-mut1 (apoE4 (L261A, W264A, F265A, L268A, V269A)) in apoE-deficient mice (apoE$^{-/-}$) corrected plasma cholesterol levels and did not cause hypertriglyceridemia. In contrast, gene transfer of apoE4-mut2 (apoE4 (W276A, L279A, V280A, V283A)) did not correct hypercholesterolemia and induced mild hypertriglyceridemia. ApoE-induced hyperlipidemia was corrected by co-infection with a recombinant adenovirus expressing human lipoprotein lipase. Both apoE4 mutants caused only a small increase in hepatic very low density lipoprotein-triglyceride secretion. Density gradient ultracentrifugation analysis of plasma and electron microscopy showed that wild-type apoE4 and apoE4-mut2 displaced apoA-I from the high density lipoprotein (HDL) region and promoted the formation of discoidal HDL, whereas the apoE4-mut1 did not displace apoA-I from HDL and promoted the formation of spherical HDL. The findings indicate that residues Leu-261, Trp-264, Phe-265, Leu-268, and Val-269 of apoE are responsible for hypertriglyceridemia and also interfere with the formation of HDL. Substitutions of these residues by alanine provide a recombinant apoE form with improved biological functions.

ApoE[1] is a polymorphic protein in humans (1). *In vitro* and *in vivo* studies have shown that apoE mutants that prevent binding of apoE-containing lipoproteins to the LDL receptor are associated with high plasma cholesterol levels and cause premature atherosclerosis in humans and experimental animals (2–4). ApoE promotes cholesterol efflux (5, 6) and thus may contribute to cell and tissue cholesterol homeostasis and protection from atherosclerosis (7, 8). ApoE is also a risk factor for Alzheimer's disease (9, 10) and may contribute to lipid homeostasis in the brain (11).

A series of recent studies used adenoviruses expressing full-length and truncated genomic apoE sequences to correct the high cholesterol profile of the apoE-deficient (apoE$^{-/-}$) mice. It was shown that overexpression of full-length apoE (by infection of mice with $1-2 \times 10^9$ pfu) did not correct the high cholesterol levels of the apoE$^{-/-}$ mice, in contrast, it increased VLDL triglyceride secretion and induced hypertriglyceridemia (12–16). Overexpression of apoE3 or apoE4 also aggravated the hypercholesterolemia in apoE2 knock-in mice (17). However the high cholesterol profile of apoE$^{-/-}$ mice or the apoE2 knock-in mice was corrected by infection with truncated apoE forms lacking different segments of the carboxyl-terminal domain (12–17). The hypertriglyceridemia induced by full-length apoE was independent of the apoE phenotype and mouse strain and could be corrected by overexpression of lipoprotein lipase (15). In normal C57BL6 mice overexpression of full-length apoE induced combined hyperlipidemia characterized by high cholesterol and high triglycerides levels, whereas truncated apoE forms did not change the plasma lipid and lipoprotein levels of these mice (13). Finally, truncated apoE forms could not correct the high cholesterol profiles of the apoE$^{-/-}$ × LDLr$^{-/-}$ double-deficient mice but did not induce hypertriglyceridemia, indicating that the carboxyl-terminal region of apoE is responsible for the hypertriglyceridemia (15, 16). Use of a series of apoE deletion mutants extending from amino acid 1 to amino acids 185, 202, 229, or 259 mapped the region responsible for the hypertriglyceridemia between amino acids 260 and 299 of apoE (12–17). This region contains two hydrophobic stretches of amino acids between residues 261–269 and 276–283.

In the present study, the hydrophobic residues of both regions were mutated to alanines and the functions of the mutant apoE forms were studied *in vivo* using adenovirus-mediated

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[1] The abbreviations used are: ApoE, apolipoprotein E; apoA-I, apolipoprotein A-I; Ad, adenovirus; ELISA, enzyme-linked immunosorbent assay; FPLC, fast pressure liquid chromatography; GFP, green fluorescence protein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LpL, lipoprotein lipase; pfu, plaque-forming unit; WT, wild type; IDL, intermediate density lipoprotein; apoE$^{-/-}$, apoE-deficient; LDLr$^{-/-}$, LDL receptor-deficient.

gene transfer. This analysis showed that residues Leu-261, Trp-264, Phe-265, Leu-268, and Ala-269 can account for the apoE-induced hypertriglyceridemia. Furthermore, the 261–269 apoE sequence is responsible for displacing apoA-I from the HDL region, leading to reduction in plasma apoA-I and HDL levels.

### EXPERIMENTAL PROCEDURES

*Construction of Recombinant Adenoviruses Expressing the Wild-type and the Mutant Forms of ApoE4*—Two apoE4 mutants were generated (apoE4-mut1 (apoE4 (L261A, W264A, F265A, L268A, V269A)) and apoE4-mut2 (apoE4 (W276A, L279A, V280A, V283A))) using the mutagenesis kit QuikChange-XL (Stratagene). The mutagenic primers used are apoE4-mut1-s (5′-GCC TTC CAG GCC CGC **GC**C AAG AGC **GC**G **GC**C GAG CCC **GC**G **GC**G GAA GAC ATG CAG CGC-3′), apoE4-mut1-a (5′-GCG CTG CAT GTC TTC C**GC** C**GC** GGG CTC G**GC** C**GC** GCT CTT G**GC** GCG GGC CTG GAA GGC-3′), apoE4-mut2-s (5′-GAC ATG CAG CGC CAG **GC**G GCC GGG **GC**G **GC**G GAG AAG **GC**G CAG GCT GCC GT-3′), and apoE4-mut2-a (5′-GCC CAC GGC AGC CTG C**GC** CTT CTC C**GC** C**GC** CCC GGC C**GC** CTG GCG CTG CA-3′). The nucleotides mutated in various codons are shown in bold. In both mutagenic reactions, the vector pGEM7-apoE4 (14) containing Exons II, III, and IV of the human apoE was used as a template. Following 18 cycles of PCR amplification of the template DNA, the PCR product was treated with DpnI to digest plasmids containing methylated DNA in one or both of their strands. The reaction product consisting of plasmids containing newly synthesized DNA carrying the mutations of interest was used to transform competent XL-10 blue bacteria cells (Stratagene). Ampicillin-resistant clones were selected, and plasmid DNA was isolated from these clones and subjected to sequencing to confirm the presence of the point mutations.

The recombinant adenoviruses were constructed as described (14) using the Ad-Easy-1 system where the adenovirus construct is generated in bacteria BJ-5183 cells (18). Correct clones were propagated in RecA DH5α cells. The recombinant adenoviral vectors were linearized with PacI and used to infect 911 cells (19). Following large scale infection of human embryonic kidney 293 cell cultures, the recombinant adenoviruses were purified by two consecutive CsCl ultracentrifugation steps, dialyzed, and titrated (14). Usually, titers of ~2–5 × 10^10 pfu/ml were obtained.

*Cell Culture Studies*—Human HTB13 cells (SW1783, human astrocytoma) grown to confluence in medium containing 10% fetal calf serum were infected with AdGFP-E4 or the adenoviruses expressing the mutant apoE forms AdGFP-E4-mut1 and AdGFP-E4-mut2 at a multiplicity of infection of 5. Twenty-four-hours postinfection, cell were washed twice with phosphate-buffered saline, and fresh serum-free medium was added. After 24 h of incubation, medium was collected and analyzed by enzyme-linked immunosorbent assay (ELISA) and SDS-PAGE for apoE expression.

*Animal Studies*—Female apoE-deficient mice 4–6-weeks-old were used in these studies. Groups of 8–10 female mice were injected intravenously through the tail vein with a dose of 2 × 10^9 pfu. Blood was obtained from the tail vein after a 4-h fast preceding adenoviral injection and 2, 3, 4, 5, and 6 days postinfection. Aliquots of plasma were stored at 4 and −20 °C.

*RNA Analysis*—To assess the expression of apoE4, apoE4-mut1, and apoE4-mut2 in infected mice, at least 3 mice from each group were sacrificed at 5 days postinfection. Livers were collected from individual animals, frozen in liquid nitrogen, and stored at −80 °C. Total RNA was isolated from the livers and analyzed for apoE mRNA expression by Northern blotting and quantitated by phosphorimaging (13).

*FPLC Analysis and Lipid Determination*—For FPLC analysis of serum samples, 12 μl of serum were diluted 1:5 with phosphate-buffered saline, and loaded onto a Superose 6 column in a SMART micro FPLC system (Amersham Biosciences), and eluted with phosphate-buffered saline. A total of 25 fractions of 50-μl volume each were collected for further analysis. Triglycerides and cholesterol were determined using the GPO-Trinder Kit (Sigma) and CHOL-MPR3 kit (Roche Applied Science), according to the manufacturer's instructions. The triglyceride and cholesterol concentrations of the serum and the FPLC fractions were determined spectrophotometrically at 540 and 492 nm, respectively, as described previously (14).

*Quantification of Human ApoE*—Human apoE concentrations were measured using sandwich ELISA (12–15).

*Rate of VLDL Triglyceride Production in C57/BL6 Mice Infected with Different ApoE Forms*—VLDL triglyceride secretion was determined following infection of C57BL6 mice with 2 × 10^9 pfu of adenoviruses
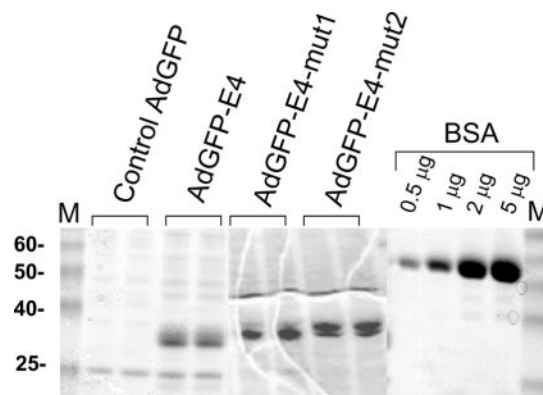


FIG. 1. **SDS-PAGE analysis of culture medium of HTB-13 cells infected with control adenoviruses and adenoviruses expressing WT apoE4, apoE4-mut1, and apoE4-mut2.** *M* indicates protein markers of different molecular mass (New England Biolabs). ApoE levels can be assessed by comparison with the intensity of the bands of samples containing 0.5–5 μg of bovine serum albumin.

expressing either WT apoE4, apoE4-mut1, or the control AdGFP adenovirus. Four days postinfection, mice were fasted for 4 h and then injected with Triton WR-1339 at a dose of 500 mg/kg of body weight, using a 15% solution (w/v) in 0.9% NaCl (Triton WR-1339 has been shown to completely inhibit VLDL catabolism (20)). Serum samples were isolated 20, 40, 60, and 90 min after injection with Triton WR-1339. Serum triglycerides were measured, and the rate of VLDL-triglyceride secretion expressed in mg/dl/min was determined as described (14).

*Statistical Analysis*—Comparison of data from two groups of mice were performed using the Student's *t* test.

*Density Gradient Ultracentrifugation*—To assess the ability of WT and mutant apoE forms to associate with different lipoproteins, 0.3 ml of serum from mice infected either with the control adenovirus AdGFP or adenoviruses expressing the WT apoE4, apoE4-mut1, or apoE4-mut2 were brought to a volume of 0.5 ml with phosphate-buffered saline and adjusted to a density of 1.23 g/ml with KBr. This solution was then overlaid with 1 ml of 1.21 g/ml KBr, 2.5 ml of 1.063 g/ml KBr, 0.5 of 1.019 g/ml KBr, and 0.5 ml of saline. The mixture was centrifuged for 22 h in a SW-41 rotor at 30,000 rpm. Following ultracentrifugation, 10 fractions of 0.5 ml were collected and analyzed by SDS-PAGE.

*Electron Microscopy*—Aliquots of the fractions from equilibrium density gradient centrifugation after dialysis against ammonium acetate and carbonate buffer were stained with sodium phosphotungstate, visualized in the Phillips CM-120 electron microscopy (Phillips Electron Optics, Eindhoven, Netherlands), and photographed as described previously (11). The photomicrographs were taken at ×75,000 magnification and enlarged three times.

### RESULTS

*The Full-length ApoE4 and the Mutant ApoE Forms ApoE4-mut1 and ApoE4-mut2 Are Secreted Efficiently by HTB-13 Cells*—To test the expression and the relative levels of secretion of the mutant apoE4 forms apoE4-mut1 and apoE4-mut2 in comparison to wild-type apoE4, HTB-13 cells that do not synthesize endogenous apoE were infected with recombinant adenoviruses expressing apoE4, or apoE4-mut1, or apoE4-mut2, or the control adenovirus AdGFP at a multiplicity of infection of 5. Analysis of the culture medium by SDS-PAGE (Fig. 1) and sandwich ELISA showed that apoE4, apoE4-mut1, and apoE4-mut2 are secreted efficiently at comparable levels (in the ranges of 130 and 170 μg of apoE/ml, respectively, 24-h postinfection).

*Residues Leu-261, Trp-264, Phe-265, Leu-268, and Val-269 Are Responsible for the ApoE-induced Hypertriglyceridemia*—We used adenovirus-mediated gene transfer in apoE^−/− mice to assess the effects of the wild-type apoE4 and the two mutants, apoE4-mut1 or apoE4-mut2, forms on the induction of hyperlipidemia *in vivo*. The apoE^−/− mice were infected with either the control adenovirus AdGFP or the recombinant adenoviruses expressing the wild-type apoE4 or the mutant forms
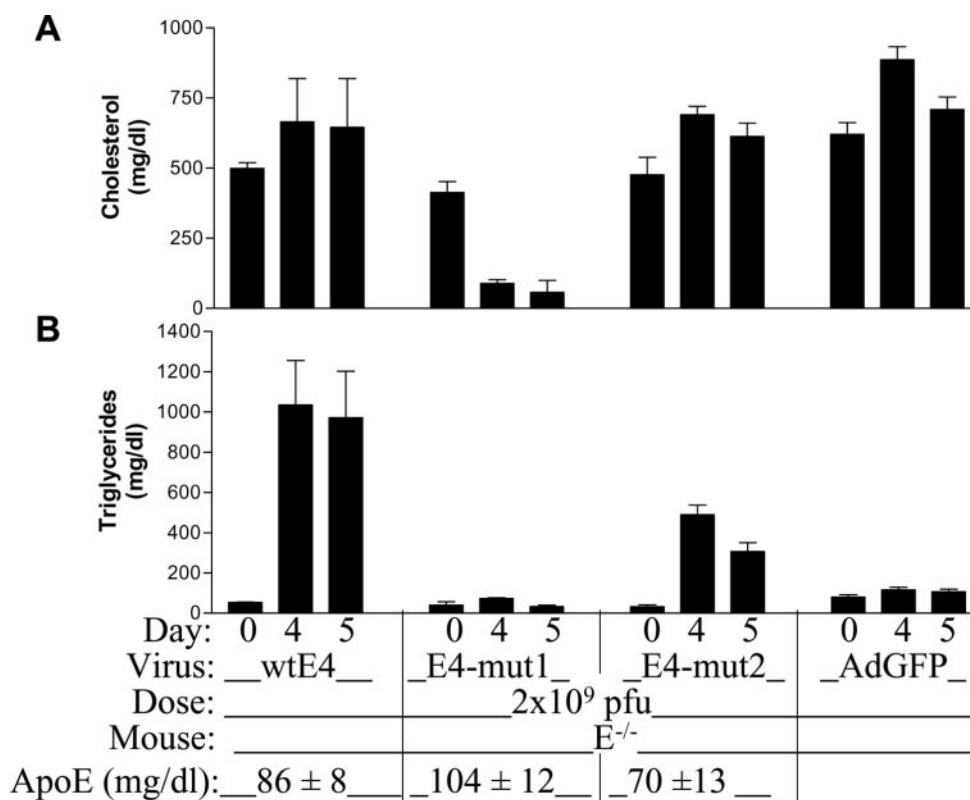
*Mechanism of ApoE-induced Dyslipidemia*



FIG. 2. **Plasma cholesterol, triglyceride, and apoE levels of apoE$^{-/-}$ mice infected with recombinant adenoviruses expressing apoE4, apoE4-mut1, apoE4-mut2, or the control AdGFP virus.** Mice were infected at least in triplicate with the indicated doses of recombinant virus, and serum samples were isolated and analyzed for cholesterol (*A*) and triglyceride levels (*B*) on the indicated days postinfection, as described under "Experimental Procedures." ApoE levels were determined on day 5 postinfection.

apoE4-mut1, which contains the point mutations L261A, W264A, F265A, L268A, V269A, and apoE4-mut2, which contains the point mutations W276A, L279A, V280A, V283A, and blood samples were collected 4 and 5 days postinfection and analyzed for plasma lipids levels. This analysis showed that the infection of mice with $2 \times 10^9$ pfu of recombinant adenovirus expressing the apoE4 or apoE4-mut2 did not alter significantly the plasma cholesterol levels 4 or 5 days postinfection and induced hypertriglyceridemia, as compared with the mice infected with the control virus (Fig. 2) and non-infected mice (data not shown). ApoE4-mut2 overexpression resulted in mild hypertriglyceridemia as compared with the wild-type apoE4 (Fig. 2). In contrast, the infection of mice with recombinant adenovirus expressing apoE4-mut1 at a dose of $2 \times 10^9$ greatly reduced plasma cholesterol levels 4 or 5 days postinfection and did not cause hypertriglyceridemia (Fig. 2).

The expression of apoE4, apoE4-mut1, and apoE4-mut2 was assessed in mice from each group 5 days postinfection by Northern blotting and apoE mRNA was quantitated by phosphorimaging. This analysis showed that apoE mRNA levels in the three groups were similar (Fig. 3). However, only apoE4-mut1 cleared efficiently the cholesterol of apoE-deficient mice without induction of hypertriglyceridemia, whereas the full-length apoE4 and the apoE4-mut2 did not correct the cholesterol levels of the apoE$^{-/-}$ mice and induced hypertriglyceridemia (Fig. 2).

*ApoE4 and ApoE4-mut2 Overexpression Results in Accumulation of Triglyceride-rich VLDL Particles, Whereas Overexpression of ApoE4-mut1 Clears VLDL*—FPLC analysis of plasma from adenovirus-infected mice showed that in mice expressing apoE4 or apoE4-mut1 5 days postinfection, cholesterol and triglyceride levels were high and were distributed predominantly in the VLDL region (Fig. 4, *A*, *C*, *D*, and *F*). In

contrast, in mice infected with AdGFP-E4-mut1, cholesterol and triglycerides were low and were distributed in all lipoprotein fractions (Fig. 4, *B* and *E*). As an additional control, the infection of mice with $2 \times 10^9$ pfu of the control virus AdGFP did not result in any change in the cholesterol and triglyceride profiles of the apoE$^{-/-}$ mice (data not shown).

*ApoE4-mut1 and ApoE4-mut2 Have a Modest Effect on the Rate of Hepatic VLDL Triglyceride Secretion*—The rate of hepatic VLDL triglyceride secretion in the plasma was determined following an injection of Triton WR-1339 5 days after the infection with the recombinant adenoviruses. It was found that, consistent with previous findings (12–16), the rate of triglyceride secretion increased 6.5-fold in mice infected with adenoviruses expressing WT apoE4 as compared with mice infected with AdGFP control. In mice infected with adenoviruses expressing either apoE-mut1 or apoE4-mut2, the rate of VLDL triglyceride secretion increased 1.9-fold as compared with mice infected with the control adenoviruses but was only 27% of the rate of VLDL secretion observed in mice infected with the apoE4-expressing adenovirus (Fig. 5). The findings suggest that residues Leu-261, Trp-264, Phe-265, Leu-268, Val-269, or residues Trp-276, Leu-279, Val-280, and Val-283 of the human apoE have a major effect on the secretion of hepatic triglycerides and when they are altered to the less hydrophobic alanines, the rate of triglyceride secretion is diminished.

*Co-expression of Full-length ApoE4 or ApoE4-mut2 and Lipoprotein Lipase Normalizes Lipid Levels in ApoE$^{-/-}$ Mice*—To test the potential insufficiency in the activity of lipoprotein lipase in the induction of hypertriglyceridemia, apoE$^{-/-}$ mice were co-infected with $2 \times 10^9$ pfu of the adenovirus-expressing apoE4, apoE4-mut1, or apoE4-mut2 and $5 \times 10^8$ pfu of adenovirus-expressing human lipoprotein lipase. This treatment corrected both the hypertriglyceridemia and the hypercholester-
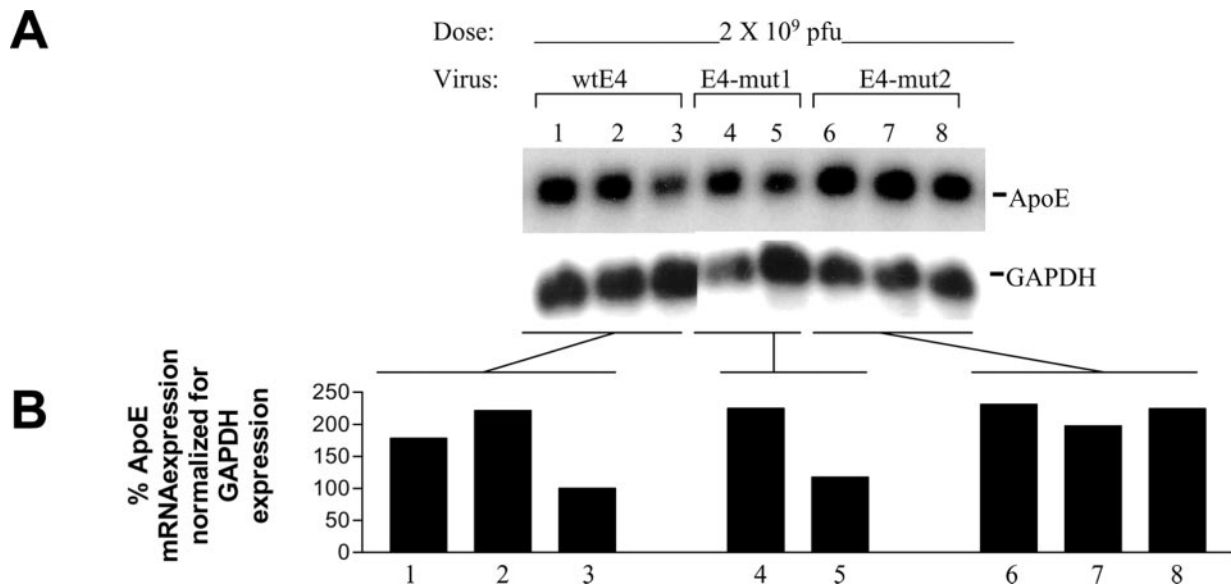
FIG. 3. **Steady-state hepatic apoE mRNA levels in the livers of individual mice infected with adenoviruses expressing apoE4, apoE4-mut2, or apoE4-mut1.** Total RNA was isolated from livers of infected mice 5 days postinfection and analyzed by Northern blotting and phosphorimaging for the expression of apoE and glyceraldehyde-3-phosphate dehydrogenase (*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 apoE4–2, apoE4-mut1 or WT apoE4. *B*, apoE mRNA levels quantified by PhosphorImager using the ImageQuant program (version 4.2A), were normalized for glyceraldehyde-3-phosphate dehydrogenase mRNA levels and reported in the format of a bar graph for each mouse.

olemia that occurs in mice treated with apoE4 or apoE4-mut2 alone (Fig. 6, *A*, *B*, *G*, and *H*). Co-infection of apoE4-mut1 with the adenovirus expressing human lipoprotein lipase decreased slightly the plasma cholesterol levels and had no significant effect on plasma triglyceride levels (Fig. 6, *D* and *E*). These findings indicated that under conditions of overexpression of apoE4 or apoE4-mut2, the endogenous lipoprotein lipase activity is rate-limiting for the lipolysis and clearance of VLDL. The combined findings in Fig. 2, *A* and *B*, Fig. 4, *B* and *E*, Fig. 5, and Fig. 6, *D* and *E*, suggest that apoE4-mut1 reduces the plasma lipid levels of apoE$^{-/-}$ mice, because it does not affect significantly hepatic VLDL-triglyceride secretion and does not have a negative effect on the activity of lipoprotein lipase.

*Effects of Overexpression of Lipoprotein Lipase on Plasma ApoE Levels in Mice Infected with Adenoviruses Expressing ApoE4, ApoE4-mut2, and ApoE4-mut1*—The lipoprotein lipase treatment had different effect on plasma apoE levels in mice treated with apoE4 as compared with mice treated with either apoE4-mut2 or apoE4-mut1 (Fig. 6, *A–I*). Thus, the apoE levels were reduced from an average value of ~125 mg/dl in mice infected with the apoE4-expressing adenovirus, 2–6 days postinfection, to an average value of ~10 mg/dl in mice infected with apoE4- and lipoprotein lipase-expressing adenoviruses (Fig. 6*C*). The lipoprotein lipase treatment reduced apoE levels from an average value of ~150 mg/dl in mice treated with apoE4-mut2 alone to an average value of ~40 mg/dl in mice treated with apoE4-mut2 and lipoprotein lipase (Fig. 6*I*). It is interesting that the greatest levels of apoE on days 2 and 3 postinfection with the apoE4-mut2-expressing adenovirus are associated with mild hypertriglyceridemia (Fig. 6, *H* and *I*). In contrast, the apoE levels of apoE4-mut1 remained high (average value of ~170 mg/dl) with or without treatment with the LpL-expressing adenovirus (Fig. 6*F*).

*ApoE4 and ApoE4-mut2, but Not ApoE4-mut1, Displace ApoA-I from the HDL Region and Promote Formation of Discoidal HDL Particles*—To establish the ability of apoE4, apoE4-mut1, and apoE4-mut2 to associate with different lipoproteins, 300 µl of serum from mice infected with either recombinant adenoviruses expressing apoE4, apoE4-mut1, or apoE4-mut2 were fractionated by density gradient ultracentrifugation.

Fractions of different densities were isolated and analyzed by SDS-PAGE followed either by staining with Coomassie Brilliant Blue stain or by Western blotting using anti-apoE antibodies. It was found that both the full-length apoE4, and the apoE4-mut1 and apoE4-mut2 mutants, associate with lipoproteins that float in the HDL region and to a lesser extent with particles in the LDL, IDL, and VLDL regions. Remarkably however, overexpression of both WT apoE4 and apoE4-mut2 resulted in displacement of apoA-I from HDL, whereas the overexpression of apoE4-mut1 does not displace apoA-I from the HDL density region (Fig. 7). We estimated, based on the SDS-PAGE analysis, that the apoA-I levels of mice infected with the apoE4-mut1 appear to be similar to those of apoE$^{-/-}$ mice, and over 90% of apoA-I was found in the HDL following density gradient ultracentrifugation (Fig. 7, compare *A* with *E*).

Electron microscopy analysis of the fraction 6–8 containing apoA-I showed that overexpression of WT apoE4 or apoE4-mut2 was associated with the formation of discoidal HDL particles, whereas the expression of apoE4-mut1 at similar levels was associated with the formation of spherical HDL particles (Fig. 8, *B–D*). The differences in the biogenesis and catabolism of VLDL and HDL in apoE$^{-/-}$ mice that overexpress apoE4, apoE4-mut2, and apoE4mut1 are summarized in Fig. 9.

## DISCUSSION

Previous *in vitro* experiments have shown that residues 260–269 of apoE are important for the binding of apoE to lipids and lipoproteins (11). Deletion of these residues diminished greatly the ability of the truncated apoE to solubilize multilamellar dimyristoyl-L-α-phosphatidylcholine vesicles. Further deletion of residues 230–299 or 203–299 and 166–299 eliminated completely the ability of apoE to solubilize multilamellar dimyristoyl-L-α-phosphatidylcholine vesicles (11). Thus it is possible that the carboxyl-terminal 260–299 amino acids of apoE may be involved in the initial association of apoE with phospholipid, a process that may be required for the formation of apoE-containing lipoproteins. Whether the association of apoE with phospholipids *in vivo* requires participation of ABCA1 remains to be established. Once apoE is lipoprotein-bound, it may be taken up by the LDL receptor.
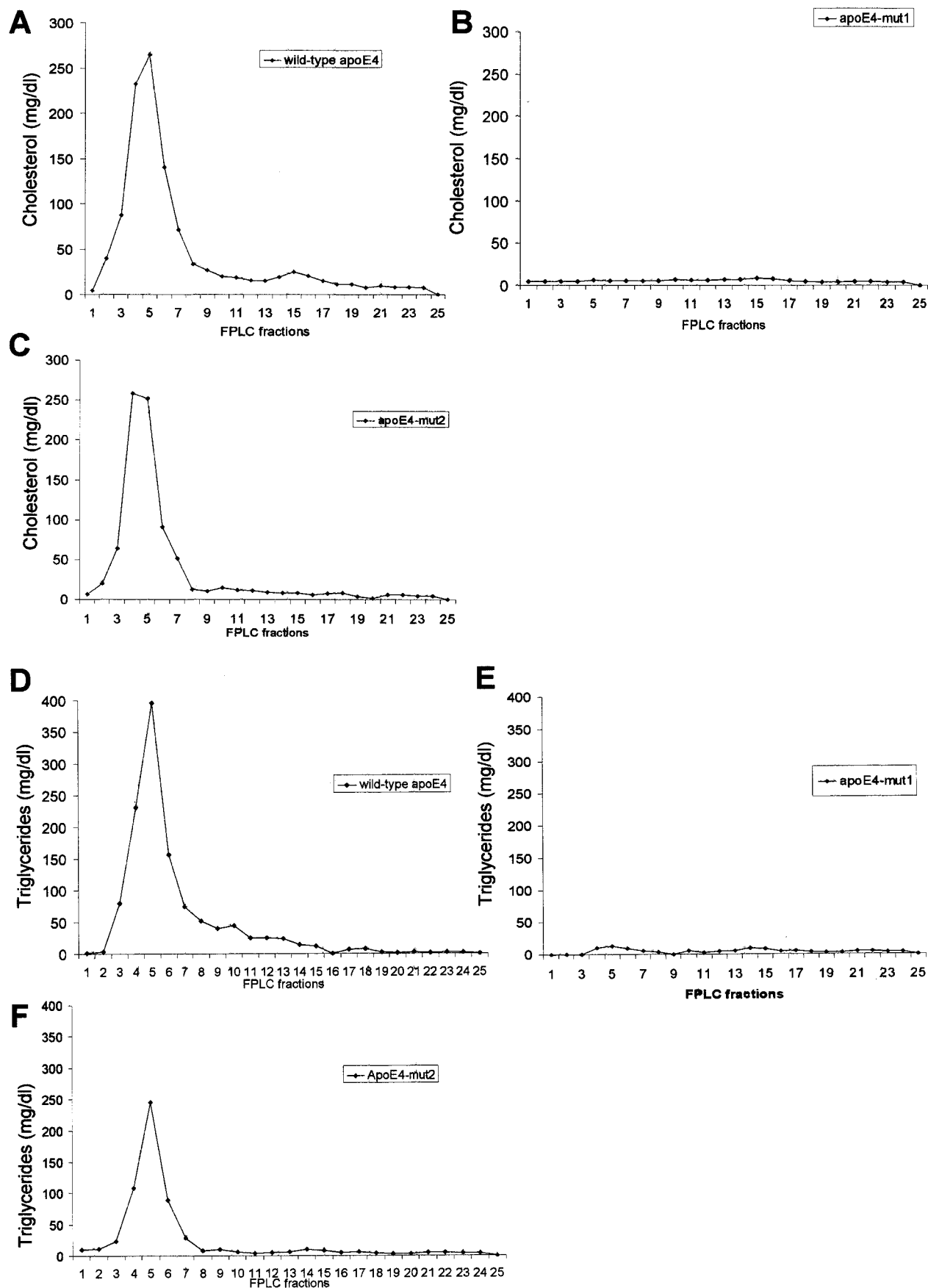
FIG. 4. **FPLC profiles of serum cholesterol (*A–C*) and triglycerides (*D–F*) of adenovirus-infected mice.** Serum samples were obtained from uninfected apoE-deficient mice or mice infected with $2 \times 10^9$ pfu of the recombinant adenoviruses expressing WT apo-E4 (*A* and *D*), apoE4-mut1 (*B* and *E*), or apoE4-mut2 (*C* and *F*) 5 days postinfection and were fractionated by FPLC. The cholesterol levels (*A–C*) and triglyceride levels (*D–F*) of each FPLC fraction were determined as described under "Experimental Procedures."
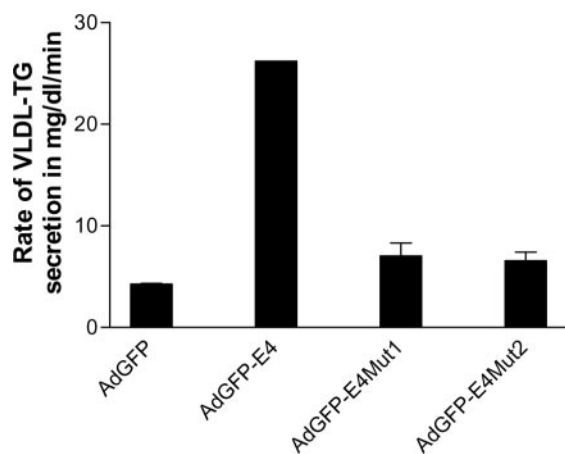
FIG. 5. **Hepatic VLDL-triglyceride (*TG*) secretion analysis in mice infected with either the control AdGFP adenovirus or recombinant adenoviruses expressing WT apoE4, apoE4-mut1, and apoE4-mut2.** Triton WR-1339 (500 mg/kg of body weight) was injected into 3 fasted mice/virus group. Serum samples were collected at 20, 40, 60, and 90 min after the injection with the detergent. 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 *versus* time was generated. The rate of VLDL-triglyceride secretion expressed in mg/dl/min was calculated from the slope of the linear graph for each individual mouse. The mean ± S.D. of the individual rates of VLDL-triglyceride secretion/ virus group are presented in the form of *bar graphs*.

The apoE-LDL receptor interactions control plasma cholesterol levels and confer protection from atherosclerosis (2). The contribution of receptors other than the LDL receptor in the clearance of apoE-containing lipoprotein remnants was previously assessed by studies in apoE$^{-/-}$ × LDLr$^{-/-}$ double-deficient mice (15). These studies have shown that neither the full-length apoE2 or apoE4 nor the truncated apoE2–202 or apoE4–202 corrected the high cholesterol profiles of the apoE$^{-/-}$ × LDLr$^{-/-}$ double-deficient mice (15). These data and other observations with full-length apoE[2] suggest strongly that apoE-mediated lipoprotein clearance in mice is carried out mainly by the LDL receptor (15). In the absence of this receptor, lipoprotein receptor-related protein, other apoE recognizing lipoprotein receptors, and heparan sulfate proteoglycans (22, 23) are not sufficient to clear the lipoprotein remnants, which accumulate in the plasma of the double-deficient mice (15). In addition to the role of apoE in cholesterol homeostasis in circulation, plasma apoE levels correlate with plasma triglyceride levels in humans (24).

Hypertriglyceridemia is also induced in mice by overexpression of human apoE (12–15, 25). However, our recent studies have shown that hypertriglyceridemia did not occur when mice were infected with adenoviruses expressing truncated apoE forms lacking the 260–299 carboxyl-terminal domain (12–16). This set of experiments also showed that when the truncated apoE forms were co-expressed with the full-length apoE forms, they had a dominant effect and normalized the cholesterol levels of the apoE$^{-/-}$ mice (15).

These findings suggested that when full-length apoE is bound to triglyceride-rich VLDL particles, its receptor binding domain may be masked, thus preventing the direct apoE-mediated clearance of the VLDL particles prior to lipolysis. In contrast, when truncated apoE is bound to triglyceride-rich VLDL particles, its receptor binding domain may be exposed and may allow direct clearance of the VLDL particle (15).

The current study was designed to map the residues in the carboxyl-terminal region of apoE, which are responsible for

hypertriglyceridemia. The rationale was that identification of these residues may lead to the generation of a recombinant apoE form with improved biological functions. We have focused on two regions of apoE between residues 260 and 299, which contain hydrophobic amino acids. The first region includes amino acids Leu-261, Trp-264, Phe-265, Leu-268, and Val-269, and the second region includes amino acids Trp-276, Leu-279, Val-280, and Val-283. A BLAST search of NCBI data base (www.ncbi.erlm.NIH.gov) showed that both regions are highly conserved among mammalian species. An *in vivo* adenovirus-mediated gene transfer of the two apoE mutants established unequivocally that the hydrophobic residues of apoE between amino acids 261–269 can account to a large extent for the induction of hypertriglyceridemia. Hypertriglyceridemia did not occur in mice infected with an adenovirus expressing apoE4-mut1 where these residues were changed into alanines. In contrast, infection of mice with an adenovirus expressing apoE4-mut2, where the hydrophobic residues between amino acids 276 and 283 were substituted by alanines, resulted in milder hypertriglyceridemia as compared with mice infected with the apoE4-expressing adenovirus.

It appears that the hydrophobic residues within the 261–269 as well as the 276–283 regions, affect the secretion of VLDL triglycerides. Mutations of these residues into alanines reduced the rate of hepatic VLDL triglyceride secretion to 27% of that caused by wild-type apoE4.

Our data also showed that an increase in the levels of the plasma lipoprotein lipase by co-infection with recombinant adenoviruses expressing the human lipoprotein lipase corrected the apoE-induced dyslipidemia in apoE$^{-/-}$ mice that overexpress full-length apoE4. This finding suggests that under the condition of apoE overexpression the activity of lipoprotein lipase becomes rate-limiting for the clearance of the hypertriglyceridemic VLDL. Substantial but less severe hypertriglyceridemia is also observed by the overexpression of apoE4-mut2, which is also corrected by co-infection with the lipoprotein lipase-expressing adenovirus. The difference in the severity of the hypertriglyceridemia between WT apoE4 and apoE4-mut2 may be related to the increased VLDL triglyceride secretion caused by the WT apoE4.

An important clue on the nature of the hypertriglyceridemic lipoprotein particles that accumulate in the plasma of the mice is provided by the clearance of apoE in mice co-infected with wild-type or mutant forms of apoE- and LpL-expressing adenoviruses. ApoE was cleared in mice treated with apoE4 and LpL, partially cleared in mice treated with apoE4-mut2 and LpL, and was unaffected in mice treated with apoE4-mut1 and LpL. The average apoE levels with or without treatment with lipoprotein lipase 2–6 days postinfection changed from ~125 mg/dl to ~10 mg/dl for apoE4, ~150 mg/dl to ~40 mg/dl for apoE4-mut2, and remained the same (~170 mg/dl) for apoE4-mut1. The findings indicate that wild-type apoE4 resides on triglyceride-rich lipoprotein particles and following the hydrolysis of the triglycerides of these particles by LpL, apoE4 is recognized and cleared as a component of the remnant particles by the LDL-receptor. It appears that the majority of the mutant apoE4-mut1 may reside in another population of triglyceride-poor particles that are not cleared by the LDL receptor. Finally, the apoE4-mut2 may be partitioned in triglyceride-rich particles that are processed by LpL and cleared by the LDL receptor and triglyceride-poor particles that are not cleared by the LDL receptor. It is possible that the mutations introduced in apoE4-mut1 and to a lesser extent in apoE4-mut2 may have promoted the formation of triglyceride-poor apoE-containing lipoprotein particles that accumulate in the HDL region of these mice.

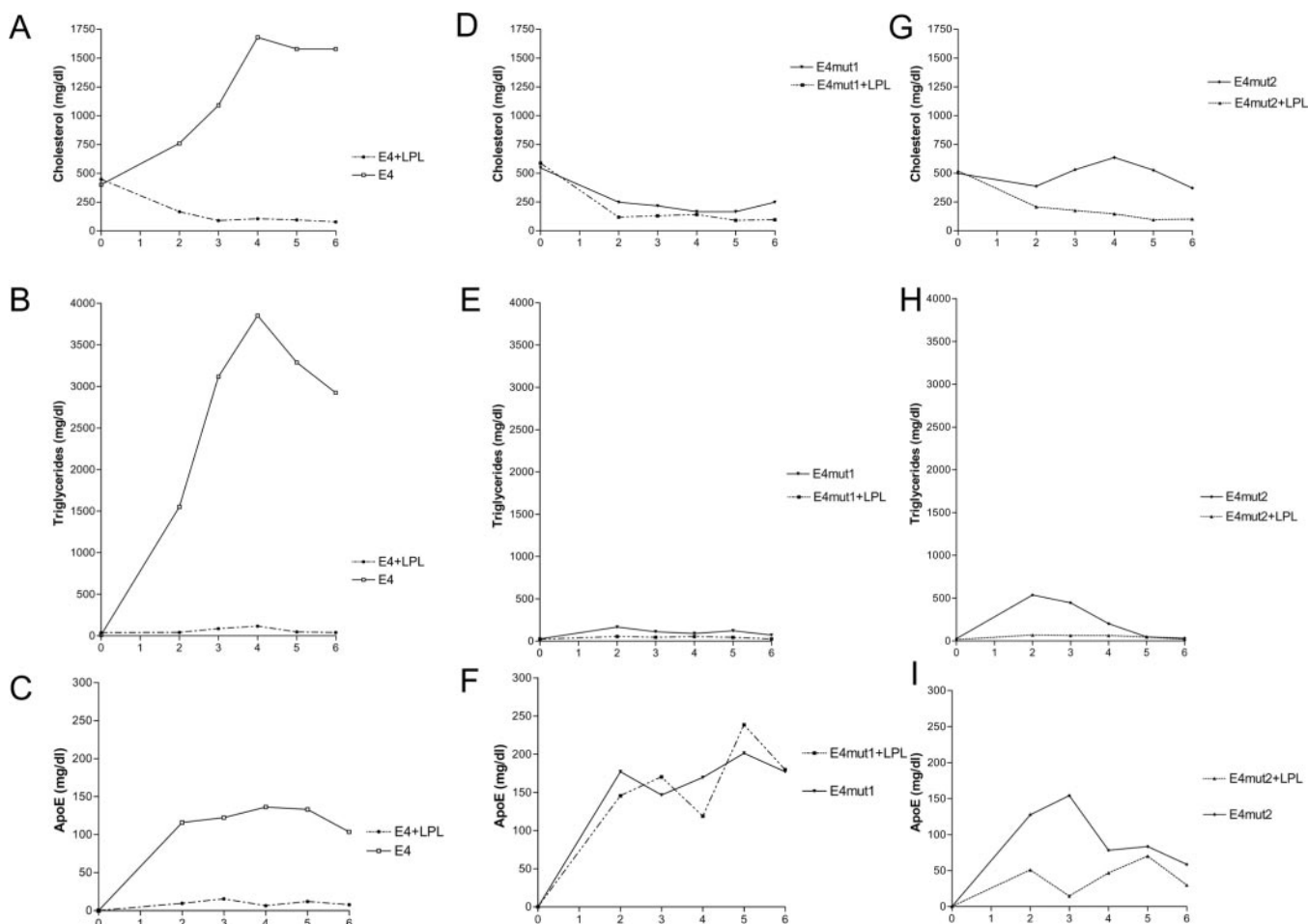A very significant finding of this study is that apoE4 and

FIG. 6. **Plasma cholesterol, triglyceride, and apoE levels of apoE$^{-/-}$ mice infected with recombinant adenoviruses expressing either full-length apoE4, apoE4-mut2, or apoE4-mut1 alone or a mixture of apoE4, apoE4-mut2, or apoE4-mut1 and human lipoprotein lipase, determined 2–6 days postinfection.** Mice were infected with the indicated doses of the recombinant adenoviruses, and serum samples were isolated and analyzed for plasma cholesterol (*A*, *D*, and *G*), triglyceride (*B*, *E*, and *H*), and apoE levels (*C*, *F*, and *I*) on days 0, 2, 3, 4, 5, and 6 postinfection, as described under "Experimental Procedures."
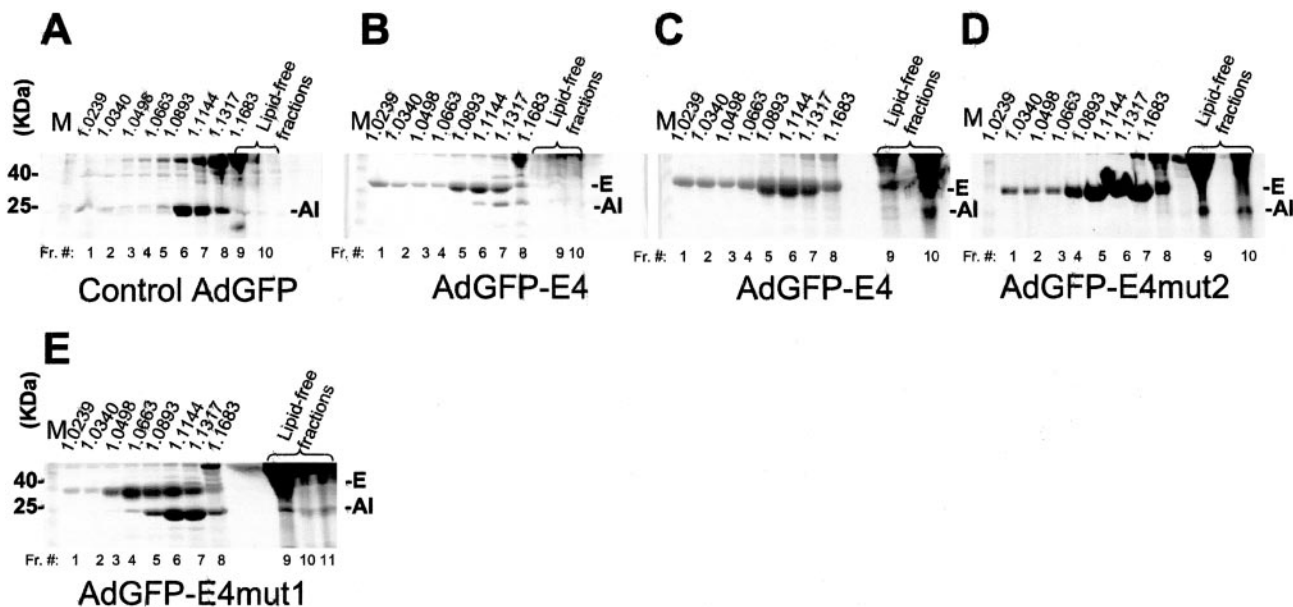


FIG. 7. **Distribution of apoE in different lipoprotein fractions following density gradient ultracentrifugation.** Plasma obtained from apoE$^{-/-}$ mice expressing WT apoE4, apoE4-mut1, or apoE4-mut2, and mice infected with control adenoviruses were fractionated by density gradient ultracentrifugation and analyzed by SDS-PAGE as described under "Experimental Procedures." *A*, analysis of plasma obtained from mice infected with control adenovirus AdGFP. *B* and *C*, analyses of plasma obtained from apoE$^{-/-}$ mice infected with WT apoE4. The concentrations of plasma apoE (*E*) in mice analyzed in *B* and *C* are 60 and 233 mg/dl, respectively. The fraction numbers and the density of each fraction and the positions of apoA-I and apoE are shown in each panel. *M* indicates protein markers of different $M_r$s. Note that depending on the levels of apoE expression, apoA-I (*AI*) is either drastically reduced (*B*) or totally displaced (*C*) from HDL. *D* and *E*, analyses of apoE$^{-/-}$ mice infected with apoE4-mut2, and apoE4-mut1, respectively.

FIG. 8. **Electron microscopy analysis of the HDL fractions obtained from plasma of mice infected with the control adenovirus AdGFP (*A*) or adenoviruses expressing WT apoE4 (*B*), apoE4-mut2 (*C*), and apoE4-mut1 (*D*).** Pooled HDL fractions 6–8 shown in Fig. 7, *A*, *C*, *D*, and *E* were used for electron microscopy analyses.
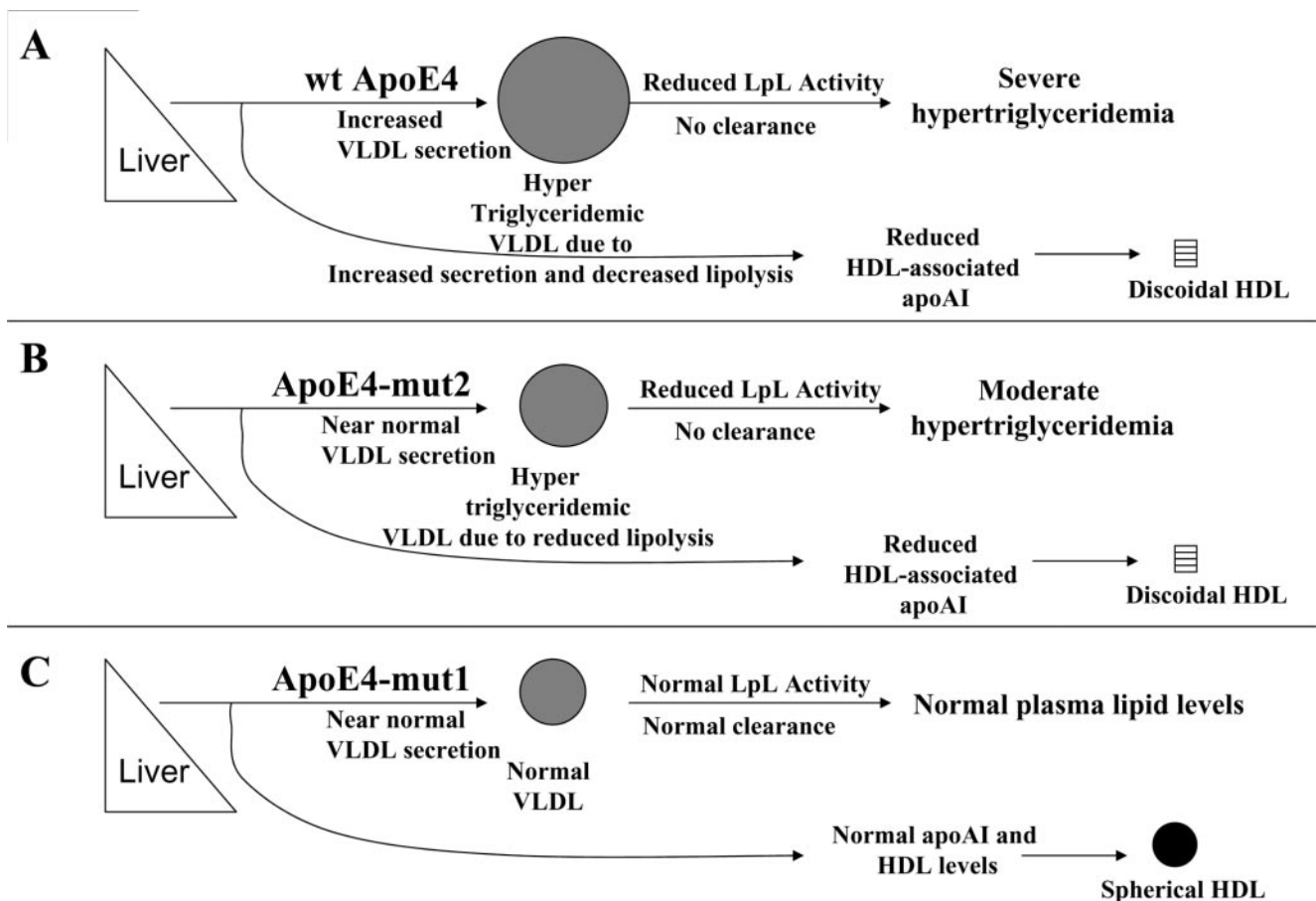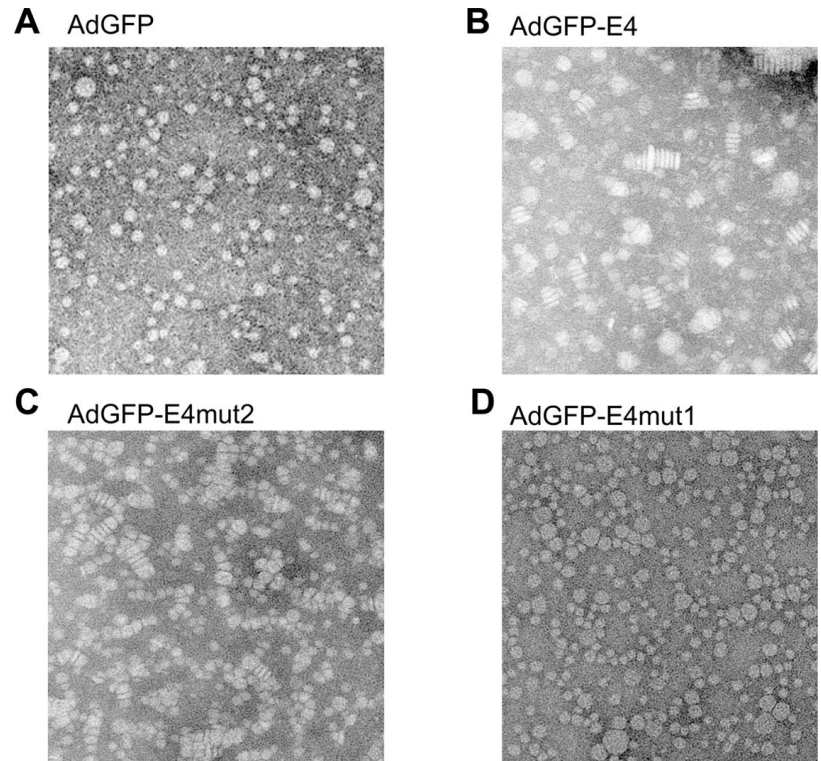


FIG. 9. **Schematic representation summarizing differences in the biosynthesis and catabolism of VLDL and HDL in apoE$^{-/-}$ mice overexpressing WT apoE4 (*A*) or the two mutants apoE4-mut2 (*B*) and apoE4-mut1 (*C*).**

apoE4-mut2 (W276A, L279A, V280A, and V283A) displaced apoA-I from HDL and promoted the formation of discoidal HDL. In contrast, apoE-mut1 (L261A, W264A, F265A, L268A, and V269A) did not displace apoA-I from the HDL region and did not affect the formation of spherical HDL particles. The findings suggest that when WT apoE4 or apoE4-mut2 is over-

expressed, they may influence the formation or the stability of HDL. The effect of the apoE mutations on the biosynthesis and catabolism of VLDL and HDL is summarized in Fig. 9, *A–C*.

At the present time, it is not clear whether apoE overexpression interferes with the biogenesis of HDL through a pathway that involves the ABCA1 transporter or whether it affects its stability by displacing apoA-I from the surface of HDL. Both processes are expected to reduce the levels of HDL. The undesirable property of WT apoE to reduce plasma HDL levels can be overcome in the recombinant apoE4-mut1, were the hydrophobic residues in the 261–269 region were mutated into alanines.

The ability of recombinant apoE forms such as apoE4-mut1 to clear cholesterol without inducing hypertriglyceridemia or interfering with the formation of spherical HDL, makes them attractive therapeutic agents to correct remnant removal disorders. Therapeutic forms of apoE may involve pure recombinant protein associated with liposomes and potential gene therapy in the future.

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