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Domains of Apolipoprotein E Contributing to Triglyceride and Cholesterol Homeostasis *in Vivo*

CARBOXYL-TERMINAL REGION 203–299 PROMOTES HEPATIC VERY LOW DENSITY LIPOPROTEIN-TRIGLYCERIDE SECRETION*

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Apolipoprotein (apo) E has been implicated in cholesterol and triglyceride homeostasis in humans. At physiological concentration apoE promotes efficient clearance of apoE-containing lipoprotein remnants. However, high apoE plasma levels correlate with high plasma triglyceride levels. We have used adenovirus-mediated gene transfer in apoE-deficient mice ($E^{-/-}$) to define the domains of apoE required for cholesterol and triglyceride homeostasis *in vivo*. A dose of 2×10^9 plaque-forming units of apoE4-expressing adenovirus reduced slightly the cholesterol levels of $E^{-/-}$ mice and resulted in severe hypertriglyceridemia, due to accumulation of cholesterol and triglyceride-rich very low density lipoprotein particles in plasma. In contrast, the truncated form apoE4–202 resulted in a 90% reduction in the plasma cholesterol levels but did not alter plasma triglyceride levels in the $E^{-/-}$ mice. ApoE secretion by cell cultures, as well as the steady-state hepatic mRNA levels in individual mice expressing apoE4 or apoE4–202, were similar. In contrast, very low density lipoprotein-triglyceride secretion in mice expressing apoE4, but not apoE4–202, was increased 10-fold, as compared with mice infected with a control adenovirus. The findings suggest that the amino-terminal 1–202 region of apoE4 contains the domains required for the *in vivo* clearance of lipoprotein remnants. Furthermore, the carboxyl-terminal 203–299 residues of apoE promote hepatic very low density lipoprotein-triglyceride secretion and contribute to apoE-induced hypertriglyceridemia.

Apolipoprotein (apo)[1] E is the ligand for several cell receptors and promotes the catabolism of apoE-containing lipoprotein remnants by the liver (1–5). Mutations in apoE that prevent binding of apoE-containing lipoproteins to the LDL receptor and possibly other receptors and heparan sulfate proteoglycans are associated with type III hyperlipoproteinemia and premature atherosclerosis (6–14).

Studies in human patients with type III hyperlipoproteinemia and in animal models with apoE deficiency or defective apoE forms (15–24) showed that apoE is required for the clearance of cholesterol ester-rich lipoprotein remnants found in the VLDL and IDL region (15–27). The accumulation of such remnants in plasma is associated with premature atherosclerosis (15, 17–19). ApoE may also be involved in cholesterol efflux processes (28–31). These functions of apoE contribute to cell and tissue cholesterol homeostasis (28–31) and may explain why, when expressed locally in macrophages or endothelial cells, apoE protects from atherosclerosis (32–34).

In humans, plasma apoE levels correlate with plasma triglyceride levels (26). Animal studies have shown that increased plasma apoE levels inhibit lipolysis of triglyceride-rich lipoproteins *in vivo* and result in hypertriglyceridemia (35–39). Lipolysis of VLDL *in vitro* could be partially restored by the addition of apoCII (35, 36). Other studies have shown that overexpression of apoE also stimulates hepatic VLDL-triglyceride production *in vivo* (39) and in cell cultures (35), possibly by promoting the assembly and/or secretion of apoB-containing lipoproteins. In contrast, lack of apoE is associated with decreased VLDL-triglyceride secretion (40). Such studies provide compelling evidence that apoE contributes both to cholesterol as well as triglyceride homeostasis *in vivo*.

To identify the domains of apoE that contribute to cholesterol and triglyceride homeostasis *in vivo*, we used adenovirus-mediated gene transfer in apoE-deficient mice. We have found that overexpression of full-length apoE4 is associated with high cholesterol and triglyceride levels, whereas overexpression of apoE4–202 normalizes the cholesterol levels of apoE-deficient mice and does not trigger hypertriglyceridemia. ApoE secretion by cells, hepatic apoE mRNA expression, and association of apoE with VLDL are not affected by the apoE truncation. In contrast, apoE4, but not apoE4–202, greatly increases hepatic

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[1] The abbreviations used are: apo, apolipoprotein; Ad, adenovirus; ELISA, enzyme-linked immunosorbent assay; FPLC, fast pressure liquid chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescence protein; HDL, high density lipoprotein; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; VLDL, very low density lipoprotein; pfu, plaque-forming units; PBS, phosphate-buffered saline; LDL, low density lipoprotein; LDLR, LDL receptor; LRP-LDL receptor-related protein; IDL, intermediate density lipoproteins; bp, base pair; WT, wild type.

TABLE I
Oligonucleotides used in overlap extension PCR

Bold underlined bases represent the mutated codon.

Oligo name	Oligo sequence
OUTPR1-S	5′-GCT GGG TGC AGA CAC TGT CTG AGC-3′ (contains a 5′ *Ngo*MI site)
OUTPR2-A	5′-CGC AGC CGC TCG CCC CAG CAG GCC T-3′ (contains a 3′ *Bst*EII site)
Internal-S	5′-C GGC CAG CCG **TGA** CAG GAG CGG G-3′
Internal-A	5′-C CCG CTC CTG **TCA** CGG CTG GCC G-3′

VLDL-triglyceride secretion. Our findings suggest that truncated forms of apoE may find useful gene therapy applications in the future in correcting remnant removal disorders.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Adenoviruses Expressing ApoE4 and ApoE4–202—The construction of pUC-apoE4 cDNA has been described previously (41). pUC-apoE4–202 was generated by overlap-extension PCR that resulted in mutagenesis of codon 203 (GTA) to a stop codon (TAA) using pUC-apoE4 as a template and the four sets of oligonucleotides indicated in Table I as primers. The set of external primers OUTPR1-S (sense) and OUTPR2-A (antisense) correspond to nucleotides encoding amino acids 103–111 and 208 -215 of apoE, respectively, and contain the restriction sites *Ngo*MI and *Bst*EII, respectively. The set of mutagenic oligonucleotides extending 10 residues 5′ and 10 residues 3′ of codon 203 has been altered in its sequence to a stop TAA codon (Table I). The PCR-based mutagenesis of codon 203 involved two separate amplification reactions. The first reactions used the 5′ external primer and the antisense mutagenic primer (Internal-A, Table I) covering codon 203. The second reaction used the 3′ external primer and the sense mutagenic primer (Internal-S, Table I) covering codon 203. An aliquot of 4% of the volume of each PCR was mixed, and the sample was amplified by the 5′ and the 3′ external primers. The amplified fragment was then digested with *Ngo*MI and *Bst*EII and was used to replace the WT sequence of the pUC-E4 plasmid. To incorporate the PCR-generated mutations to exon IV of the apoE gene, a two-step procedure was followed. The *Eco*RI fragment of the human apoE4 gene, which includes the entire exon IV sequence, was cloned into the *Eco*RI site of the pBS vector to generate the vector pBlue-exIV. The pUC-apoE4–202 plasmid was digested with *Sty*I/*Bbs*I, and the mutated sequence was exchanged for the WT sequence of the pBlue-E4-exIV plasmid to generate plasmid pBlue-E4–202-exIV plasmid.

The recombinant viruses were constructed using the Ad-Easy-1 system where recombinant adenovirus construct is generated in bacteria BJ-5183 cells (42). The 1507-bp *Msc*I-*Eco*RI fragment of apoE genomic DNA (nucleotides 1853–3360), which contains exons 2 and 3, was cloned into the *Sma*I-*Eco*RI sites of pGEM7 vector, resulting in the pGEM7-apoE-Ex II, III vector. The 1911-bp *Eco*RI fragment of apoE4 or apoE4–202 gene (that contains the stop mutation at codon 203) was then excised from pBlue-E4-Ex IV or the pBlue-E4–202-Ex IV vector, respectively, and cloned into the *Eco*RI site of the pGEM7-apoE-Ex II, III vector. This generated the pGEM7-apoE4g or pGEM7-apoE4g-202 vector, respectively, that contains exons II–IV of the apoE gene. The correct orientation of the 1911-bp *Eco*RI insert was checked by restriction digest with *Not*I and *Xba*I. The entire *Hin*dIII-*Xba*I fragment from pGEM7-apoE4g or pGEM7-apoE4–202-g vector was cloned into the corresponding sites of the pAd Track-cytomegalovirus adenovirus shuttle plasmid. The recombinant vector was used to electroporate BJ 5183 *Escherichia coli* cells along with the pAd Easy-1 helper vector. pAdEasy-1 contains the viral genome and the long terminal repeats of the adenovirus and allows for the formation by homologous recombination of the recombinant virus containing the gene of interest. The vector also contains the green fluorescence protein gene, which enables detection of the infection of cells and tissues by their green fluorescence. Recombinant bacterial clones resistant to kanamycin were selected and screened for the presence of the gene of interest by restriction endonuclease analysis and DNA sequencing. The viruses expressing WT apoE4 and apoE4–202 forms are designated as AdGFP-E4 and AdGFP-E4–202, respectively. Correct clones were propagated in RecA DH5α cells. The recombinant vector was linearized with *Pac*I and used to infect 911 cells. The subsequent steps involved in the generation and expansion of recombinant adenoviruses were plaque identification/isolation followed by infection and expansion in 911 cells (43). These steps were followed by a purification process involving CsCl ultracentrifugation performed twice, followed by dialysis and titration of the virus. Usually, titers of $\sim 5 \times 10^{10}$ 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 AdGFP-E4–202, at a multiplicity of infection of 20. Twenty four hours post-infection, 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 24 h of incubation, medium was collected and analyzed by enzyme linked immunoabsorbent assay (ELISA) and SDS-PAGE for apoE expression. In some experiments, 60-mm diameter cultures were labeled metabolically with 0.5 mCi of [^{35}S]methionine for 2 h, in which case medium was collected 2 h after addition of the radiolabeled amino acid for further analysis.

Association of ApoE Secreted into the Culture Medium of Cells with Plasma Lipoproteins following Density Gradient Ultracentrifugation—For this analysis, following the labeling of cells with [^{35}S]methionine, 1 ml of medium from one 100-mm diameter dish was mixed with 1 ml of lipoprotein fractions of densities (d) in the range of 1.006–1.21 g/ml, which were previously separated from plasma by density gradient ultracentrifugation, the mixture was adjusted to density 1.24 with KBr and overlaid with 4 ml each of KBr solutions of $d = 1.20$ and 1.06, and 2 ml of KBr solution of $d = 1.019$ g/ml. The mixture was centrifuged for 24 h in a SW-41 rotor at 32,000 rpm. Following ultracentrifugation, 12 1-ml fractions were collected and analyzed by SDS-PAGE and autoradiography. This analysis showed that both apoE4 and apoE4–202 can associate with exogenous lipoproteins that float in the IDL to HDL region.

Isolation of VLDL by Density Gradient Ultracentrifugation—One ml of serum sample obtained from apoE- and LDLR-deficient mice were overlaid on a KBr density composed of 1 ml of 1.21 g/ml KBr, 1 ml of 1.063 g/ml KBr, 1 ml of 1.019 g/ml KBr, and 1 ml of saline. Samples were subjected to ultracentrifugation at 30,000 rpm in an SW-55 rotor for 16 h, and then the top 1 ml of the gradient containing the VLDL fraction was isolated.

Binding of ApoE to VLDL Particles—Two hundred fifty microliters of VLDL, isolated from the plasma of apoE-deficient mice, was mixed with 750 μl of culture medium containing 15 μg of apoE4 or apoE4–202 secreted by HTB cells that were infected with AdGFP-E4 or 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—Female apoE-deficient mice 20–25 weeks old were used in these studies (44). Groups of mice were formed based on their plasma cholesterol and triglyceride levels before initiation of the experiments to ensure similar mean cholesterol and triglyceride levels in each group. The mice were injected intravenously through the tail vein with doses ranging from 5×10^8 to 1×10^{10} pfu of AdGFP (control adenovirus), AdGFP-E4, or AdGFP-E4–202 virus, as indicated. Each group contained 8–10 mice. Blood was obtained from the tail vein or retro-orbital plexus after a 4-h fast preceding adenoviral injection. On the indicated days after injection (0, 4 5, 8, and 12), blood was collected into a CB300 or CB1000 blood collection tube (Sarstedt). Aliquots of plasma were stored at 4 and −20 °C. One or more animals from each group was sacrificed on each of the indicated days so that mRNA expression in the mouse liver could be analyzed.

FPLC Analysis—For FPLC analysis of serum samples, 12 μl of serum were diluted 1:5 with PBS. Then sample was loaded onto a Sepharose 6 column in a SMART micro FPLC system (Amersham Pharmacia Biotech) and eluted with PBS. A total of 25 fractions of 50-μl volume each were collected for further analysis.

Triglyceride and Cholesterol Analysis—Ten μl of serum sample were diluted with 40 μl of phosphate-buffered saline (PBS), and 7.5 μl of the dilute sample were analyzed for triglycerides and cholesterol using the GPO-Trinder Kit (Sigma) and CHOL-MPR3 kit (Roche Molecular Biochemicals), according to the manufacturer's instructions. Triglyceride

and cholesterol concentrations were determined spectrophotometrically at 540 and 492 nm, respectively. Triglyceride analysis of the FPLC fractions was performed using the TG Buffer (Sigma), and concentrations were determined spectrophotometrically at 492 nm according to the manufacturer's instructions. Cholesterol analysis of the FPLC fractions was performed as described above for the serum samples.

Quantification of Human ApoE—Serum human apoE4 concentrations were measured by using sandwich ELISA (45). Affinity-purified polyclonal goat anti-human apoE antibodies were used for coating microtiter plates, and polyclonal goat anti-human apoE coupled to horseradish peroxidase was used as the secondary antibody. The immunoperoxidase procedure was employed for the colorimetric detection of apoE at 450 nm, using tetramethylbenzidine as substrate. Pooled plasma from healthy human subjects with known apoE level was used as a standard.

RNA Isolation and Hybridization Analysis—Total RNA was isolated from livers of mice five days post infection using the RNA Easy solution (RNA Insta-Pure, Eurogentec Belgium) according to the manufacturer's instructions. For Northern blot analysis, RNA samples (15 μg) were denatured and separated by electrophoresis on 1.0% formaldehyde-agarose gels. RNA was stained with ethidium bromide to verify integrity and equal loading and then transferred to GeneScreen Plus (PerkinElmer Life Sciences). RNA was cross-linked to the membrane by UV irradiation (Stratalinker, Stratagene) at 0.12 J/cm^2 for 30 s. Probes, prepared by random priming, were used as described previously, and 2.0×10^6 cpm/ml ^{32}P-labeled DNA was employed. Quantitation by scanning densitometry was performed using a Molecular Dynamics PhosphorImager (model 400B). ApoE mRNA expression was normalized for GAPDH mRNA levels and reported in the form of a bar graph. Experiments were performed in triplicate, and data are reported as mean value ± S.D.

Rate of VLDL-Triglyceride Production in Mice Infected with Different ApoE Forms—To determine the effects of the apoE truncations on hepatic VLDL-triglyceride secretion, four apoE-deficient mice for each group were infected with a dose of 2×10^9 pfu of AdGFP-E4 and 1×10^{10} pfu of the AdGFP-E4–202 or the control AdGFP viruses, respectively. Four days post-infection, 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 inhibit completely VLDL catabolism (46)). Serum samples were isolated 5, 10, 20, 30, 40, 50, and 60 min after injection with Triton WR1339. As control, serum samples were isolated 1 min immediately after the injection with the detergent. Serum triglyceride levels were determined as described under the "Experimental Procedures," and a linear graph of serum triglyceride *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. Then slopes were grouped together and reported as mean ± S.D. in the form of a bar graph.

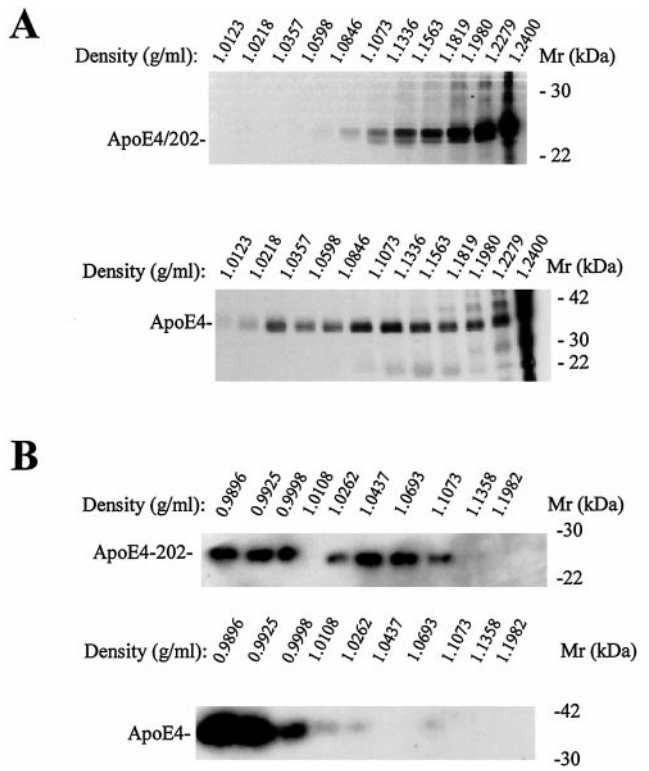

FIG. 1. ***A* and *B*, flotation properties of apoE4 and apoE4–202.** *A,* separation by density gradient ultracentrifugation of apoE-containing lipoprotein secreted by HTB-13 cells infected at a multiplicity of infection of 20 with apoE-expressing recombinant adenoviruses. The figure shows autoradiograms of different density fractions analyzed by SDS-PAGE, following density gradient ultracentrifugation in the presence of purified lipoproteins as described under "Experimental Procedures." (*M,* indicates proteins of known molecular mass purchased from New England Biolabs). The densities of the different fractions are as indicated. *B,* Western blot analysis of apoE4 and apoE4–202 fractions bound to VLDL particles from apoE and LDLR double-deficient mice. Approximately 15 μg of apoE4 and apoE4–202 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 blotting analysis as described under "Experimental Procedures."

RESULTS

Expression and Flotation Properties of ApoE Secreted by HTB-13 Cell Cultures Infected with Control and Recombinant Adenovirus Containing ApoE4 or ApoE4–202—HTB-13 cells that do not synthesize endogenous apoE were infected with recombinant adenoviruses expressing apoE4 or apoE4–202, designated AdGFP-E4 and AdGFP-E4–202, respectively, at a multiplicity of infection of 20. Analysis of the culture medium by SDS-PAGE and sandwich ELISA showed that both apoE4 and apoE4–202 are secreted efficiently at comparable levels in the range of 60–80 μg of apoE per ml 24 h after infection with AdGFP-E4 or AdGFP-E4–202. To study the flotation properties of apoE4 and apoE4–202, 100-mm diameter dishes of HTB-13 cultures that were infected with AdGFP-E4 or AdGFP-E4–202, respectively, were metabolically labeled with 0.5 mCi of [^{35}S]methionine for 2 h, and then 1 ml of medium from each dish was mixed with ~1 mg of lipoprotein fractions of densities (*d*) in the range of 1.006–1.21 g/ml, which were previously separated from plasma by density gradient ultracentrifugation. The mixture was subjected again to density gradient ultracentrifugation, and 12 fractions were collected and fractionated on SDS-PAGE, followed by autoradiography. This analysis showed that the majority of apoE4 and a considerable fraction of apoE4–202 floats in the $d > 1.21$ g/ml lipoprotein fraction, although a large quantity of apoE-202 was also found in the lipid-poor or lipid-free fraction (Fig. 1*A*). To establish further the ability of apoE4 and apoE4–202 to associate with VLDL, 15 μg of apoE4 or apoE4–202 were mixed with VLDL fractions isolated from the plasma of apoE- and LDLR-deficient mice by ultracentrifugation, and the mixtures were incubated at 37 °C for 30 min. The mixture was then subjected to density gradient ultracentrifugation. The amounts of the free and lipoprotein-associated apoE were assessed by fractionation on SDS-PAGE followed by Western blot analysis for apoE. As shown in Fig. 1*B*, both the full-length apoE4 and the truncated apoE4–202 associate with particles with densities in the VLDL to LDL region. ApoE4 in this analysis is found in the VLDL to IDL region, and apoE4–202 appears to associate with particles in the VLDL to HDL_3 region. The data indicate that both the wild-type apoE4 as well as the truncated apoE form, apoE4–202, have the ability to associate with pre-existing lipoprotein particles, a process that is required for receptor-mediated lipoprotein clearance.

The Carboxyl-terminal 203–299 Segment of ApoE Contributes to Hypertriglyceridemia in ApoE-deficient Mice—To assess the effects of apoE4 and apoE4–202 on hyperlipidemia *in vivo*, apoE-deficient mice ($E^{-/-}$) were infected with the recombinant adenoviruses AdGFP-E4 or AdGFP-E4–202, respectively. To

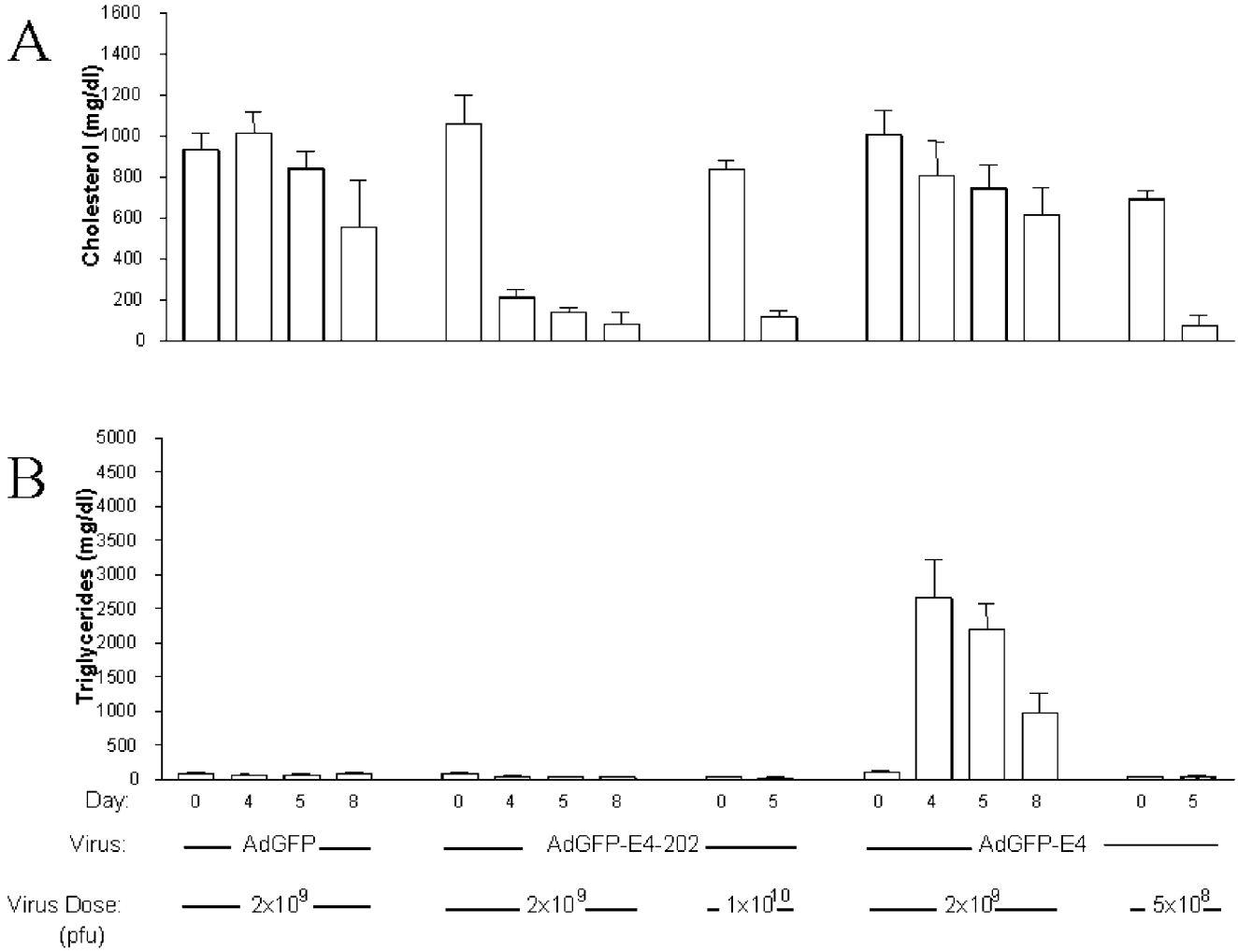

FIG. 2. ***A* and *B*, cholesterol (*A*) and triglyceride (*B*) levels of $E^{-/-}$ mice infected with the control adenovirus AdGFP or recombinant adenoviruses expressing apoE4 or apoE4–202.** Mice were infected in triplicate with the indicated doses of recombinant virus, and serum samples were isolated and analyzed for cholesterol (*A*) and triglyceride levels (*B*) on the indicated days after infection as described under "Experimental Procedures."

assess potential nonspecific effects of virus infection, some mice were infected with the control AdGFP virus. Analysis of plasma lipid levels showed that the infection of mice with 2×10^9 pfu of the apoE4-adenovirus did not result in a significant reduction in the plasma cholesterol levels, as compared with the cholesterol levels of mice infected with the control virus and non-infected mice (Fig. 2*A*). In addition, infection with 2×10^9 pfu of the apoE4 adenovirus induced severe hypertriglyceridemia (Fig. 2*B*). When expression of apoE4 on day 8 post-infection was reduced or when the dose used for infection was decreased to 5×10^8 pfu, hypertriglyceridemia was also reduced or eliminated (Fig. 2, *A* and *B, right side*). In contrast to the mice infected with the adenovirus expressing the wild-type E4, mice that were infected with 2×10^9 or even 10^{10} pfu of the adGFP-E4–202 adenovirus that expresses the truncated form apoE4–202 had normal cholesterol levels and did not develop hypertriglyceridemia (Fig. 2, *A* and *B, middle part*). The control virus AdGFP did not appear to have significant effects on the cholesterol and triglyceride levels as compared with the levels of non-infected apoE-deficient mice, ruling out the possibility of nonspecific effects of the infection process (Fig. 2, *A* and *B, left side*).

The Hepatic ApoE4 and ApoE4–202 mRNA Levels Are Similar in Infected Mice, under Conditions of ApoE4-induced Hypertriglyceridemia—To assess the expression of apoE4 and apoE4–202 in the mice infected with AdGFP-E4 and AdGFP-E4–202, respectively, at least three infected mice from each group were sacrificed on day 5 post-infection, and their livers were collected. Total RNA was isolated from these livers and analyzed for apoE mRNA levels by Northern blot analysis. In agreement with cell culture data, where we see similar levels of apoE4 and apoE4–202 protein expression following adenovirus infection, the apoE mRNA levels in mice infected with a dose of 2×10^9 pfu AdGFP-E4 are similar to those in mice infected with either 2×10^9 pfu or 1×10^{10} pfu AdGFP-E4–202 (Fig. 3, *A* and *B*). However, apoE4–202 clears efficiently the cholesterol from the plasma of $E^{-/-}$ mice without causing hypertriglyceridemia, whereas the full-length apoE4 does not clear cholesterol from the plasma of $E^{-/-}$ mice and causes hypertriglyceridemia (Fig. 3, *C* and *D*). Thus, the different effects of apoE4 and apoE4–202 on hypertriglyceridemia most likely are not due to different levels of expression of these two apoE forms.

Cholesterol, Triglyceride, and ApoE FPLC Profiles of Plasma Isolated from Mice Infected with AdGFP-E4, AdGFP-E4–202, or the Control Virus AdGFP—FPLC analysis of plasma from adenovirus-infected mice showed that in mice expressing apoE4 5 days post-infection, cholesterol levels were high. Approximately 70% of cholesterol was distributed in VLDL and ~20% in HDL. On day 8 post-infection, the ratio of VLDL/HDL

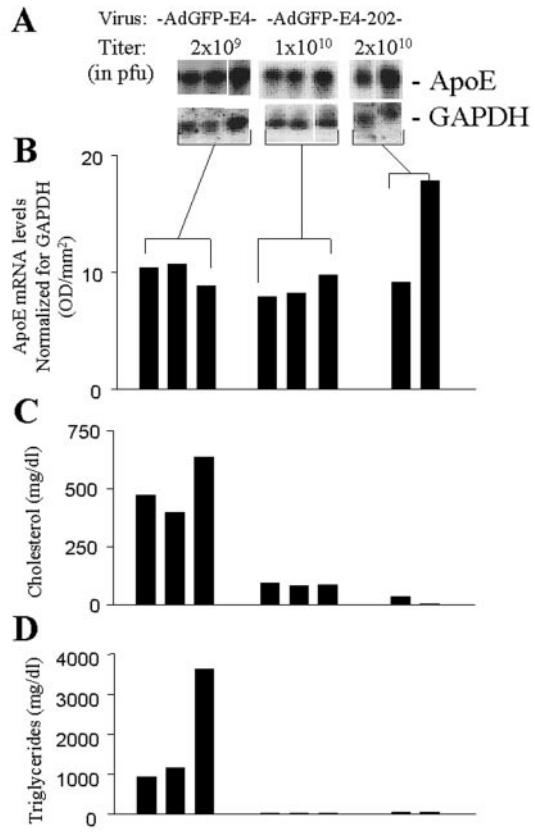

FIG. 3. ***A–D*, correlation of hepatic apoE mRNA levels with plasma cholesterol and triglyceride levels of individual mice infected with apoE4- or apoE4–202-expressing adenoviruses.** Total RNA was isolated from livers of infected mice five days after infection and analyzed by Northern blotting for the expression of apoE and GAPDH mRNA. *A* shows representative autoradiograms of Northern blot analysis of total RNA isolated from livers of mice infected with the indicated dose of the recombinant adenoviruses expressing apoE4 or apoE4–202. *B* shows apoE mRNA levels quantified by PhosphorImager using the ImageQuant program (version 4.2A), were normalized for GAPDH mRNA levels and reported in the format of a *bar graph* for each mouse. *C* shows cholesterol levels of the individual mice expressed in mg/dl. *D* shows triglyceride levels of the individual mice expressed in mg/dl.

cholesterol was ~1:1 to 2:1 (Fig. 4*A*, *upper panel*). In mice infected with AdGFP-E4–202, cholesterol was distributed in the VLDL and HDL, and the ratio VLDL cholesterol to HDL cholesterol was ~1:1 on either day, when the dose of adenovirus used was either 2×10^9 or 10^{10} pfu (Fig. 4*A*, *lower panels*). In mice infected with AdGFP-E4, as expected, triglyceride levels were very high in the VLDL fractions and barely detectable in the rest of the lipoprotein fractions, whereas in mice infected with AdGFP-E4–202 triglyceride levels were very low in all the lipoprotein fractions either 5 or 8 days post-infection (Fig. 4*B*). As an additional control, infection with 2×10^9 pfu of the control virus AdGFP, did not result in any change in the cholesterol and triglyceride profiles of the apoE-deficient mice (data not shown).

Analysis of FPLC fractions by sandwich ELISA showed that in mice infected with 2×10^9 pfu AdGFP-E4, 5 days post-infection ~50% of the total apoE was distributed in HDL and 25% in VLDL, and the remaining apoE was distributed across the other FPLC fractions. In contrast, in mice infected with 10^{10} pfu AdGFP-E4–202, apoE was uniformly distributed in all lipoprotein fractions (Fig. 5). The levels of apoE4 and apoE4–202 are similar in fractions 22–25, which represent the lipid-free apoE form (Fig. 5). This indicates that similar steady-state levels of lipid-free apoE4 and apoE4–202 exist in the plasma of mice infected with 2×10^9 pfu AdGFP-E4 and 10^{10} pfu AdGFP-E4–202, respectively. The average total plasma apoE levels, based on a pool of plasma of five mice, were 315 μg/ml for apoE4 and 13 μg/ml for apoE4–202. The apparent lower concentration of apoE4–202 in plasma and in the lipoprotein-containing fractions most likely reflects the efficient catabolism of the apoE4–202-containing lipoproteins.

Wild-type ApoE4 Increases Significantly the Rate of Hepatic VLDL-triglyceride Production as Opposed to the Truncated Form ApoE4–202 and the Control AdGFP Virus—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 decreased ~50% in mice infected with 1×10^{10} pfu of AdGFP-E4–202 and increased 10-fold in mice infected with 2×10^9 pfu of AdGFP-E4, as compared with mice infected with 1×10^{10} pfu of the control AdGFP adenovirus (Fig. 6). The findings suggest that the carboxyl-terminal region of apoE influences the rate of VLDL-triglyceride secretion and contributes to apoE-induced hypertriglyceridemia.

DISCUSSION

ApoE promotes receptor-mediated catabolism of apoE-containing lipoproteins by cell receptors (1–5). Early studies showed that mutation in apoE within the vicinity of amino acids 130–160 affected the recognition of this protein by the LDL receptor and resulted in dominant or recessive forms of type III hyperlipoproteinemia and premature atherosclerosis (6–13, 47–49). Two additional functions of apoE pertinent to triglyceride homeostasis were suggested by the analysis of human subjects and transgenic animals expressing different levels of apoE. The first function of apoE is related to secretion of VLDL-triglycerides (35, 39, 40, 50), and the second is related to the inhibition of lipolysis (25, 27, 35–38, 51). Both processes are expected to increase the plasma triglyceride levels in humans and in experimental animals (25–27, 35–38, 51). To dissect these apoE functions, we have used adenovirus-mediated gene transfer to express at similar levels either the full-length apoE4 or the truncated apoE4–202 form in apoE-deficient mice. The mice were analyzed 4–8 days post-infection for apoE expression, plasma lipid and lipoprotein profiles, and mechanisms that impede the clearance of plasma cholesterol and triglycerides when the full-length apoE4 is overexpressed.

The Amino-terminal Segment 1–202 of ApoE Can Associate with Lipoproteins and Direct Their Catabolism in Vivo

Receptor-mediated catabolism of apoE requires association of apoE with lipoprotein particles, whereas lipid-free apoE does not bind to lipoprotein receptors (1–6). Heparan sulfate proteoglycans may also be involved in apoE binding on the cell surface (52–55). Domains of apoE involved in receptor binding (47–49, 56), heparin binding (57–58), and lipid and lipoprotein binding (59, 60) have been identified *in vitro*. The receptor binding domain is found between residues 136 and 152, whereas neighboring residues may also indirectly affect receptor binding (48, 49). The amino acids 142–147 of the receptor binding domain are also involved in heparin binding. Two other heparin binding domains were found between residues 211 and 218 and 243 and 272 (57, 58). It has been proposed that binding of apoE-containing lipoproteins to heparan sulfate proteoglycans contributes to their subsequent internalization with or without the participation of LRP (2, 6, 55). Other studies suggested that the region of apoE between residues 244 and 299 contributes to the binding of apoE to lipids and lipoproteins, whereas the amino-terminal region of apoE lacks the determinants required for association with lipoproteins (60). In addi-

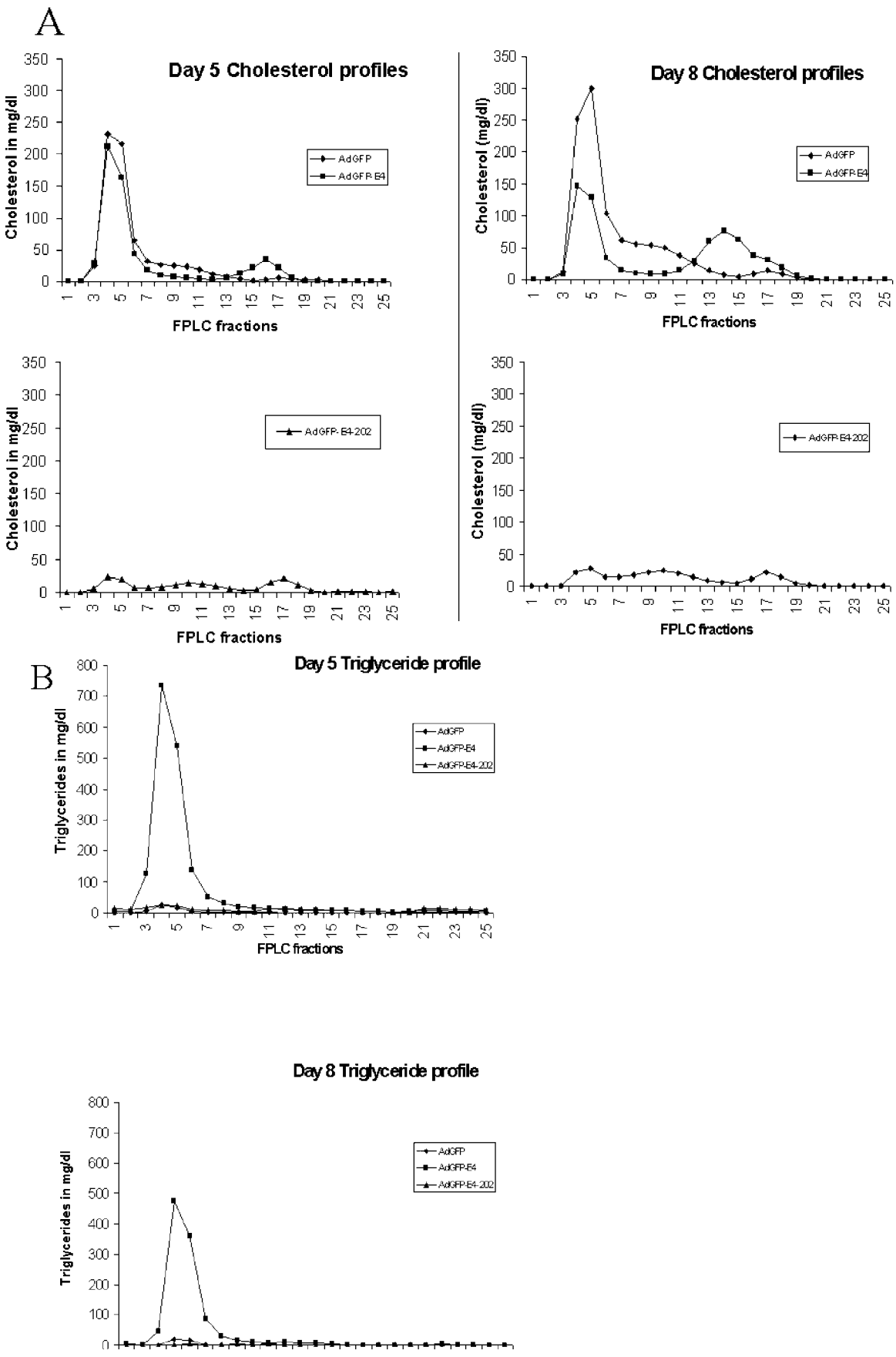

FIG. 4. ***A* and *B*, FPLC profiles of cholesterol (*A*) and triglycerides (*B*) of mice infected with apoE4- or apoE4–202-expressing adenoviruses.** Serum samples obtained from mice infected with 2×10^9 of the control virus AdGFP, or the recombinant adenoviruses expressing AdGFP-E4 or AdGFP-E4–202 on days 5 and 8 post-infection, were fractionated by FPLC, and then the cholesterol and triglycerides levels of each FPLC fraction were determined as described under "Experimental Procedures."

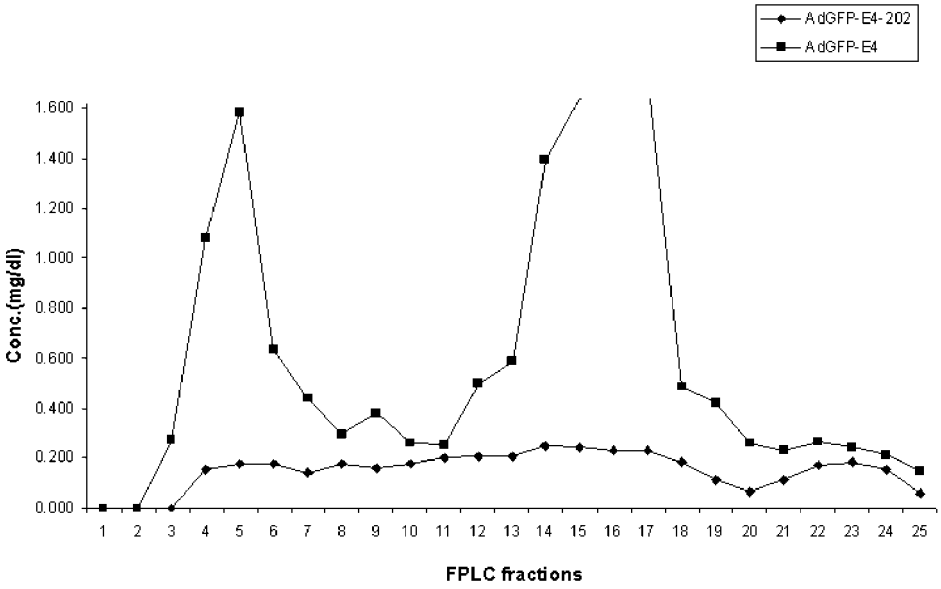

FIG. 5. **FPLC profile of plasma apoE4 in mice infected with apoE4- or apoE4–202-expressing adenoviruses.** Serum samples from mice infected with either 2×10^9 pfu apoE4-expressing adenovirus or 1×10^{10} pfu apoE4–202-expressing adenoviruses were obtained 5 days post-infection. ApoE was quantified by sandwich ELISA as described under "Experimental Procedures."

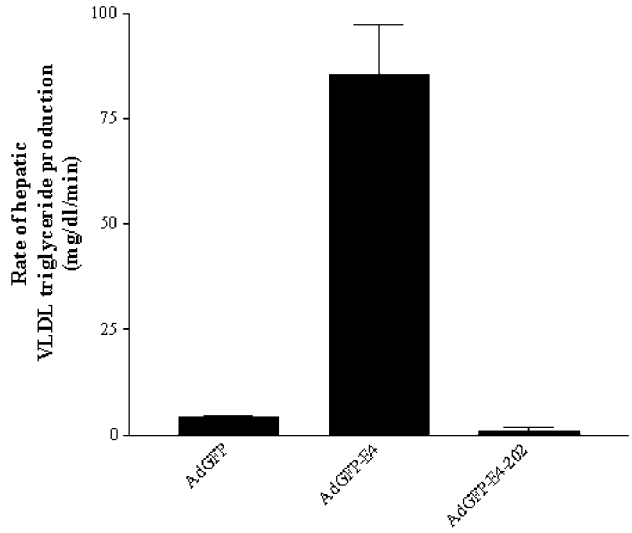

FIG. 6. **Hepatic VLDL-triglyceride production analysis in mice infected with 1×10^{10} pfu AdGFP, or 2×10^{10} pfu AdGFP-E4, or 1×10^{10} pfu AdGFP-E4–202.** Triton WR1339 (500 mg/kg body weight) was injected into three fasted mice per virus group. Serum samples were collected at 20, 40, and 60 min after the injection with the detergent. As 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 *bar graph* represents the mean ± S.D. of the individual rates of VLDL-triglyceride production per virus group.

tion, residues 267–299 were found to be important for the tetramerization of apoE (60).

The present study refines the domains of apoE required for its association with lipoproteins, a process required for their *in vivo* clearance. Our findings establish that the amino-terminal residues 1–202 of apoE contain the domains necessary for the clearance of cholesteryl ester-rich lipoprotein remnants *in vivo.* This implies that the region 1–202 of apoE contains the necessary determinants for the association of apoE with these lipoprotein remnants *in vitro.* This conclusion is supported by *in vitro* data in the present study, showing that apoE4 and apoE4–202 remain associated with VLDL and LDL particles following ultracentrifugation. The clearance of lipoprotein remnants by apoE4–202, which contains only the 142–147 heparin binding domains, is extremely efficient. Previous studies showed that the 243–272 heparin binding domain of apoE is shielded when apoE is bound to lipids and lipoproteins and thus is inactive as a ligand for heparan sulfate proteoglycans (57). It is possible that the major route of clearance of the lipoproteins containing apoE4–202, which contains only the 142–147 heparin binding domain, is via the LDL receptor and LRP pathways without the involvement of the heparan sulfate proteoglycan pathway. The potential participation of the LRP and the LDL receptor in the clearance of apoE4–202-containing remnants may make their uptake and the subsequent cholesterol clearance more efficient and thus may account for the observed efficiency of apoE4–202 in cholesterol clearance. This hypothesis is currently under investigation.

The Carboxyl-terminal Domain of ApoE Contributes to Hypertriglyceridemia

A very important finding of this study is that removal of the carboxyl-terminal region 203–299 of apoE4 prevents the development of hypertriglyceridemia following adenoviral infection. Several control experiments were performed to confirm that the different properties of apoE4 and apoE4–202 are not the result of differences in expression, secretion, or association with VLDL. Northern blot analysis of total RNA has established unequivocally that the steady-state apoE mRNA levels in mice overexpressing apoE4 that result in hypertriglyceridemia and the mRNA levels in mice overexpressing apoE4–202 that do not cause hypertriglyceridemia are very similar (Fig. 3, *A–D*). This analysis indicates that it is unlikely that decreased expression of the truncated apoE forms is responsible for this effect. In addition, cell culture experiments showed that C127 (mouse mammary tumor) cell lines stably transfected and expressing apoE4 or apoE4–202 (data not shown), or HTB-13 cell cultures infected with recombinant adenovirus, secrete similar amounts of apoE4 and apoE4–202. These observations indicate that the truncated apoE4–202 form is stable and is secreted as efficiently as its wild-type apoE4 counterpart.

Mechanisms of ApoE-induced Hypertriglyceridemia

Aside from differences in synthesis, secretion, and VLDL association between apoE4 and apoE4–202, other factors that could have contributed to the apoE-induced hypertriglyceridemia are increased hepatic VLDL-triglyceride synthesis, decreased catabolism of apoE4 as compared with apoE4–202-containing particles, and a combination of both processes.

Increased Hepatic VLDL-Triglyceride Secretion Promoted by the Carboxyl-terminal 203–99 Residues of ApoE4—The present study shows that the hepatic VLDL-triglyceride secretion, following injection of Triton WR1339, decreases by 50% in mice expressing apoE4–202 and increases 10-fold in mice expressing apoE4, as compared with the mice infected with the control virus AdGFP (Fig. 6). This implies that apoE4 contributes to hypertriglyceridemia, at least partially, by increasing VLDL-triglyceride secretion. The hypertriglyceridemic VLDL may be a poor ligand for apoE-recognizing receptors, despite the fact that it is enriched with apoE. A previous study also showed that VLDL-triglyceride secretion is reduced by 50% in $E^{-/-}$ mice (40). The findings imply that the carboxyl-terminal residues 203–299 of apoE may be directly involved in intracellular assembly and secretion of VLDL-triglycerides.

Increased VLDL Catabolism Is Mediated by Truncated ApoE Forms and Impediment of Triglyceride Hydrolysis Is Caused by Overexpression of ApoE—Work by others (60) has shown that injection of two ^{125}I-truncated apoE forms extending from residues 1 to 191 and 1 to 244, respectively, in rabbits, resulted in their fast and very efficient removal from plasma. These observations are consistent with the finding of this study, which shows that apoE4–202 contributes to the efficient clearance of apoE-containing lipoprotein remnants *in vivo*. The efficiency of apoE4–202-mediated clearance of lipoprotein remnants also results in the concomitant clearance of the apoE molecules, thus resulting in lower levels of steady-state plasma apoE4–202 as observed in this study. The steady-state levels of apoE in plasma are a function of apoE synthesis, secretion, and receptor-mediated clearance that requires association of apoE with lipoproteins. As shown in Figs. 2 and 4, mice expressing WT apoE4 do not clear VLDL particles, and this results in the accumulation of VLDL-associated apoE in the plasma of those mice (Fig. 5). In contrast, mice expressing the truncated forms apoE4–202 clear VLDL particles very efficiently, and this results in decreased cholesterol and triglyceride and apoE levels in the plasma of apoE4–202-expressing mice (Figs. 2, *A* and *B* and 5).

Aside from the intracellular effect of apoE on VLDL-triglyceride secretion that we observe, other studies (35, 36) have shown that excess of secreted apoE may displace partially the lipoprotein lipase or apoCII and thus reduce lipolysis. Preliminary analysis of the apoprotein composition of VLDL particles obtained from AdGFP-E4- and AdGFP-E4–202-infected mice by SDS-PAGE showed that other proteins present in triglyceride-rich VLDL, such as apoA-IV and apoA-I, are also displaced by full-length apoE4 but not by apoE-202 (data not shown). Analysis of the apoE structure by x-ray crystallography and computer modeling showed that the amino-terminal domains of apoE contain antiparallel helices (61, 62). It is possible that these amino-terminal helices extending from residues 23 to 185 bind to specific sites on the lipoprotein surface along with other protein particles. At a critical apoE concentration these apoE sites may be saturated, and the clearance of apoE-containing lipoproteins may be optimized. With further increases in plasma apoE concentration, specific displacement or inhibition of the function of other critical protein components of the triglyceride-rich lipoproteins may take place by the carboxyl-terminal helices, found within the 203–299 region of apoE. To the extent that lipoprotein lipase and apoCII are affected by the excess of apoE, one may expect that the rate of lipolysis of these particles will be reduced and the plasma triglyceride levels will rise.

Overall, the current study indicates that the amino-terminal 1–202 residues of apoE are sufficient for binding to lipoprotein remnants to an extent that promotes their efficient clearance *in vivo*, whereas the carboxyl-terminal 203–299 region of apoE contributes to hypertriglyceridemia. At least part of the hypertriglyceridemic effect of apoE results from increased VLDL-triglyceride secretion. This hypertriglyceridemic VLDL may be recognized poorly by apoE receptors. Hypertriglyceridemia may be further exacerbated by diminished lipolysis of VLDL. The identification of amino acid residues within the carboxyl-terminal region of apoE, which mediate the hypertriglyceridemic effect of apoE, is the subject of ongoing research.

Therapeutic Potential of Truncated ApoE Forms

Expression of apoE within a physiological range clears lipoprotein remnants, whereas overexpression results in hypertriglyceridemia. The undesirable side effect resulting from apoE overexpression diminishes significantly the therapeutic value of apoE. The inability of the truncated apoE form that lacks the carboxyl-terminal 203–299 region to induce hypertriglyceridemia, as described in this research, coupled with its intact ability to clear cholesterol, makes it an attractive candidate in future gene therapy approaches to correct remnant removal disorders.

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Domains of Apolipoprotein E Contributing to Triglyceride and Cholesterol Homeostasis *in Vivo* : CARBOXYL-TERMINAL REGION 203–299 PROMOTES HEPATIC VERY LOW DENSITY LIPOPROTEIN-TRIGLYCERIDE SECRETION

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