Apolipoprotein E Participates in the Regulation of Very Low Density Lipoprotein-Triglyceride Secretion by the Liver*

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ApoE-deficient mice on low fat diet show hepatic triglyceride accumulation and a reduced very low density lipoprotein (VLDL) triglyceride production rate. To establish the role of apoE in the regulation of hepatic VLDL production, the human APOE3 gene was introduced into apoE-deficient mice by cross-breeding with APOE3 transgenics (APOE3/*apoe*−/− mice) or by adenoviral transduction. APOE3 was expressed in the liver and, to a lesser extent, in brain, spleen, and lung of transgenic APOE3/*apoe*−/− mice similar to endogenous *apoe*. Plasma cholesterol levels in APOE/*apoe*−/− mice (3.4 ± 0.5 mM) were reduced when compared with *apoe*−/− mice (12.6 ± 1.4 mM) but still elevated when compared with wild type control values (1.9 ± 0.1 mM). Hepatic triglyceride accumulation in apoE-deficient mice was completely reversed by introduction of the APOE3 transgene. The *in vivo* hepatic VLDL-triglyceride production rate was reduced to 36% of control values in apoE-deficient mice but normalized in APOE3/*apoe*−/− mice. Hepatic secretion of apoB was not affected in either of the strains. Secretion of ³H-labeled triglycerides synthesized from [³H]glycerol by cultured hepatocytes from apoE-deficient mice was four times lower than by APOE3/*apoe*−/− or control hepatocytes. The average size of secreted VLDL particles produced by cultured apoE-deficient hepatocytes was significantly reduced when compared with those of APOE3/*apoe*−/− and wild type mice. Hepatic expression of human APOE3 cDNA via adenovirus-mediated gene transfer in apoE-deficient mice resulted in a reduction of plasma cholesterol depending on plasma apoE3 levels. The *in vivo* VLDL-triglyceride production rate in these mice was increased up to 500% compared with LacZ-injected controls and correlated with the amount of apoE3 per particle. These findings indicate a regulatory role of apoE in hepatic VLDL-triglyceride secretion, independent from its role in lipoprotein clearance.

Apolipoprotein E is an important constituent of triglyceride-rich lipoproteins such as VLDL[1] and chylomicrons and is essential for effective receptor-mediated uptake of their remnants (1). High levels of apoE delay lipoprotein lipase-mediated lipolysis of these lipoproteins (2, 3). ApoE deficiency in mice leads to elevated plasma cholesterol concentrations because of the accumulation of VLDL- and chylomicron-remnants (β-VLDL), which results from impaired hepatic uptake of these particles (4–6). As a consequence, atherosclerotic lesions rapidly develop in apoE-deficient mice (Refs. 5 and 7; for review see Ref. 8). A secretion-recapture role for apoE has been proposed in which the apoprotein is secreted by hepatocytes into the space of Disse to interact with heparan sulfate proteoglycans, followed by binding and internalization of circulating lipoproteins (9, 10). Data from *in vitro* studies indicate that apoE may also serve a function in intracellular metabolism and distribution of lipids after their uptake by macrophages (11) and hepatoma cells (12). Recent studies from our laboratory have shown that apoE deficiency leads to a 3-fold hepatic fat accumulation in mice kept on low fat chow (6, 13) and to a 50–60% reduced production of VLDL-associated triglycerides by the liver (6). Based on these results, we hypothesized that apoE may have a physiological function in the VLDL production cascade. To test this hypothesis, we investigated whether introduction of apoE into apoE-deficient hepatocytes would actually stimulate hepatic VLDL-triglyceride production in a dose-dependent fashion. Therefore, the human APOE3 gene was introduced into apoE-deficient mice, either through cross-breeding of apoE-deficient mice with transgenic mice expressing APOE3 or through adenovirus-mediated transduction with human APOE3 cDNA. The results of these studies are compatible with our hypothesis that apoE exerts a regulatory function in hepatic VLDL-triglyceride production in the mouse, independent from its role in lipoprotein uptake.

EXPERIMENTAL PROCEDURES

Animals—Transgenic mice expressing human APOE3 were generated according to Hogan *et al.* (14), using a DNA construct obtained from plasmid pJS276 (kindly provided by Dr. J. D. Smith, The Rockefeller University, New York, NY) as described previously (2). Transgenic offspring was identified by polymerase chain reaction analysis and Southern blot analysis on genomic tail-derived DNA. Six founders were obtained from which one strain, exhibiting high expression of human APOE3 in liver, was bred with C57BL/6J. Homozygous APOE3 transgenic mice of the F1 generation were cross-bred with apoE-deficient mice to obtain APOE3/*apoe*−/− mice. The resulting offspring was analyzed for the presence of apoE3 by sandwich enzyme-linked immu-

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[1] The abbreviations used are: VLDL, very low density lipoprotein; apo, apolipoprotein.

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nosorbent assay and the endogenous *apoe*−/− genotype through tail tip DNA analysis, as described earlier (7).

Mice were housed in a light- and temperature-controlled environment. Food and tap water was available *ad libitum*. The animals were fed a commercial lab chow (RMH-B, Hope Farms BV, Woerden, The Netherlands) containing 6.2% fat and approximately 0.01% cholesterol (w/w). Male mice were used throughout the study at 3–4 months of age. The animals received humane care, and experimental protocols complied with local guidelines for use of experimental animals.

Adenovirus Transductions—The generation of the recombinant adenoviral vectors expressing either human APOE3 (Ad-APOE3) (15) or the β-galactosidase gene (Ad-β-Gal) (16) under the control of the cytomegalovirus promotor has previously been described. The Ad-APOE3 was kindly provided by Dr. S. Santamarina Fojo (Betheseda, MD) and Ad-β-Gal by Dr. J. Hertz (Dallas, TX). The recombinant adenovirus was propagated and titrated in a way similar to that already described (17). For storage, the virus was supplemented with mouse serum albumin (0.2%) and glycerol (10%). The aliquots were flash-frozen in liquid N_2 and stored at −80 °C. Routine virus dilution of the stocks varied from $1–5 \times 10^{10}$/ml.

For *in vivo* adenovirus transductions, 1×10^9 to 2×10^9 plaque forming units in a total volume of 200 µl (diluted with phosphate-buffered saline) were injected into the tail vein of apoE-deficient mice. Five days after virus injection, mice were fasted for 4 h prior to measurement of VLDL-triglyceride production. Triton WR 1339 was injected intravenously, and blood samples were drawn from the tail vein at timed intervals, as described below.

Human APOE3 mRNA Measurements—Total RNA was isolated from brain, heart, kidney, liver, muscle, skin, and spleen using the RNA Instapture System (Eurogentec S.A., Seraing, Belgium). RNA samples (7.5 µg/lane) were separated by electrophoresis through a denaturing agarose gel (1% w/v) containing 7.5% formaldehyde and transferred to a nylon membrane (Hybond N, Amersham Pharmacia Biotech) according to the manufacturer's recommendations. Blots were subsequently hybridized with a ^{32}P-labeled probe of human APOE (18) at 53 °C in a solution of 50% formamide and of 18 S (19) at 65 °C in a solution containing 0.5 M Na_2HPO_4/NaH_2PO_4, 1 mM EDTA, and 7% SDS (w/v).

In a different set of experiments, the amounts of human APOE3 mRNA in livers of APOE3 transgenic mice and adenovirus-transduced mice were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The amounts of human APOE3 mRNA were related to the level of glyceraldehyde-3-phosphate dehydrogenase mRNA (20).

Immunogold Labeling and Electron Microscopy—For immunoelectron microscopic studies, livers were processed essentially as described by Hamilton *et al.* (21) for rat liver. In short, mice were anesthetized with halothane, followed by perfusion-fixation of the liver via the portal vein with freshly prepared 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Perfusion was performed at a flow rate of 1 ml/min. Following fixation, livers were sliced and washed with 6.8% sucrose in 0.1 M phosphate buffer, incubated in 2.3 M sucrose for at least 2 h at 4 °C, and then mounted on copper pins. Samples were stored in liquid nitrogen until use. Cryosections (∼80 nm) were made using an LKB-Reichert-Jung ultracryomicrotome (Leica, Rijswijk, the Netherlands) with a glass knife and were immunolabeled with goat anti-human APOE3 antibody (1:1000 dilution). Antibody binding was detected with 6-nm gold particles conjugated with rabbit anti-goat IgG (1:30 dilution). Sections were stained, covered with a methylcellulose uranylacetate mixture (0.3% uranylacetate), air dried, and stored at room temperature. Sections were examined using an EM 201 transmission electron microscope (Philips, Eindhoven, the Netherlands) operated at 80 kV.

Plasma and Liver Tissue Sampling—Groups of 5–6 mice were anesthetized with halothane. A large blood sample for determination of plasma lipids was collected by cardiac puncture. Subsequently, the liver was quickly removed, weighed, and immediately frozen in separate portions in liquid nitrogen for RNA isolation and lipid analysis, respectively. Parts of livers used for microscopical examination were stored in paraformaldehyde or slowly frozen in isopentane. Frozen sections were stained with Oil-Red-O for detection of neutral fat.

Lipid Analyses—Hepatic and plasma concentrations of triglycerides and free and total cholesterol were measured using commercial kits (Roche Molecular Biochemicals). Phospholipid concentrations in liver tissue were determined as described (6) after lipid extraction according to Böttcher *et al.* (22). The plasma concentrations of lathosterol, β-sitosterol, and campesterol were determined by capillary gas chromatography on a Hewlett Packard HP5890 gas chromatograph, as described previously (23). Plasma lipoproteins were separated by Fast Protein Liquid Chromatography (Amersham Pharmacia Biotech) on a Superose
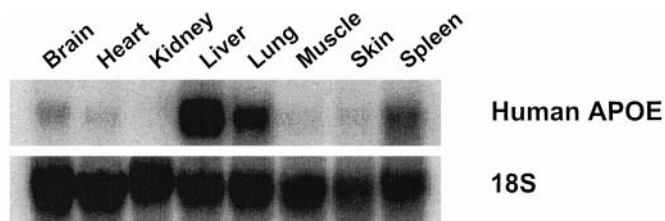

FIG. 1. **Northern blot analysis of human APOE3 gene expression in different organs of APOE3/*apoe*−/− mice.** RNA (7.5 µg) isolated from the various organs using the RNA Instapture System was used for Northern blot analysis followed by hybridization with a human APOE3 cDNA probe (*top panel*) and an 18 S probe (*bottom panel*) for standardization.

6B column as described previously (24).

Analysis of apoE3 Levels—ApoE3 levels in total plasma were measured by enzyme-linked immunosorbent assay (7). Fast protein liquid chromatography samples were subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel (Bio-Rad) according to Laemlli (25) and transferred to nitrocellulose (Amersham Pharmacia Biotech). Detection was performed by ECL-Western blotting detection reagents (Amersham Pharmacia Biotech) according to the instructions provided.

In Vivo VLDL-triglyceride Production Rate—Hepatic production of VLDL-triglycerides was measured in control, *apoe*−/−, and APOE3/*apoe*−/− mice after intravenous injection of Triton WR 1339, exactly as described (26). Mice were fasted for 16 h prior to the experiments, and 12.5 mg of Triton WR 1339 in 100 µl phosphate-buffered saline was injected via the penile vein. Tail vein blood samples were taken under light halothane anesthesia before and at 1, 2, and 3 h after Triton injection for triglyceride measurements. Liver weights and body weights were carefully recorded.

VLDL Isolation and apoB Production Measurements—Hepatic production rates of VLDL apoB-100 and of B-48 were determined according to Li *et al.* (26). 350–400 µl of plasma obtained at 3 h after Triton WR 1339 injection was adjusted to 1 ml with a NaCl/NaBr solution of density 1.019 g/ml containing 1 mM EDTA and NaN_3 and centrifuged at 120,000 rpm in a Beckman Optima™ 102.2 rotor for 100 min at 4 °C (27). The VLDL was isolated by tube slicing, and the recovered volume was measured by weight. VLDL protein was separated by SDS-polyacrylamide gel electrophoresis, simultaneously with four dilutions of human low density lipoprotein apoB (0.525, 1.05, 1.58, and 2.1 µg) prepared as described previously (28). VLDL apoB-100 and B-48 was quantified by laser densitometry (Imagemaster, Amersham Pharmacia Biotech) and comparison with standards. Three mice per group were used for apoB base-line analysis. Hepatic production rates were determined as described (26).

In Vitro Measurement of VLDL-triglyceride Secretion—Mouse hepatocyte isolation and culturing was done as described previously (6, 29). In short, the portal vein was cannulated with a 22-gauge plastic cannula. The liver was perfused with a calcium-free HBSS containing 10 mM glucose (pH 7.4), pregassed with 95% O_2/5% CO_2, at a flow rate of 4.5 ml/min. This was followed by perfusion of the liver with a collagenase solution (20 mg/125 ml calcium (5 mM)-containing Hank's balanced salt solution) until swelling of the liver was observed. Hepatocytes were gently released from the surrounding capsule and washed in Krebs buffer containing 10 mM Hepes and 10 mM glucose and with Williams' E medium. Cells were plated in 35-mm 6-well plastic dishes (Costar Corp., Cambridge, MA), precoated with collagen (Serva Feinbiochemica, Heidelberg, Germany) at a density of 1.0×10^6 cells/well in 2 ml of Williams' E medium containing insulin, fetal calf serum, dexamethason, and penicillin/streptomycin. After overnight incubation, the medium was removed, and hepatocytes were washed twice with fetal calf serum- and hormone-free (SF-HF) medium and subsequently incubated for four hours in 2 ml SF-HF medium. Cells were then incubated in SF-HF medium containing 4.4 µCi of [^{3}H]glycerol (Amersham Pharmacia Biotech; final volume, 25 µM) for 3 h with or without 0.75 mM oleate (C18:1) complexed with bovine serum albumin (final concentration, 0.25 mM) to stimulate hepatocytic lipogenesis. After a 3-h incubation period, the medium was collected and centrifuged to remove debris, and lipids were extracted as described previously (30). Hepatocytes were washed three times and scraped into 2 ml of phosphate-buffered saline for lipid extractions. Lipids from medium and hepatocytes were dissolved in chloroform with 2 mM tripalmitin added as a carrier. Triglycerides were separated from other lipids by TLC with hexane/diethylether/acetic acid (80/20/1, v/v/v) as developing solvent. Tri-
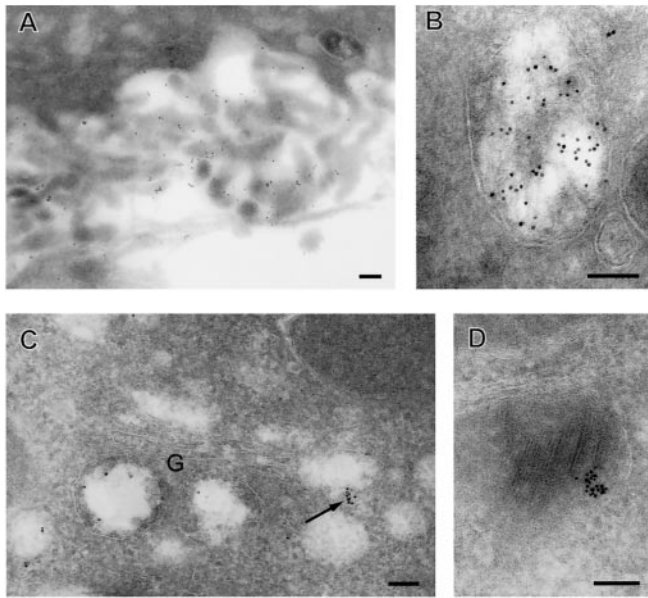
FIG. 2. **Immunoelectron microscopy of apoE3 in hepatocytes from APOE3/apoe−/− mice.** Livers were perfusion-fixed, and sections were prepared as detailed under "Experimental Procedures." ApoE3 was visualized with goat antibody against human ApoE and 6-nm gold particles conjugated to rabbit anti-goat antibody. *A*, extensive labeling of hepatocytic microvilli. *B*, multivesicular body filled with apoE-containing remnants. *C*, Golgi apparatus-associated labeling, in budding and trans-Golgi structures. The *arrow* indicates apoE labeling. *D*, peroxisomes were labeled in a characteristic, cluster-like fashion. *G* indicates Golgi. *Bars* indicate 0.1 μm.

TABLE I

Plasma lipid concentrations in chow-fed wild type (C57BL/6J), apoe−/−, and APOE3/apoe−/− mice

Blood samples were collected by cardiac puncture from halothane-anaesthetized mice after a 4-h fast. Lipid contents were measured in EDTA plasma using enzymatic kits, as outlined under "Experimental Procedures." Values represent the means ± S.D., $n = 5$ in all groups.

	Triglycerides	Total cholesterol	Free fatty acids
	mM	*mM*	*mM*
Wild type	0.20 ± 0.07	1.87 ± 0.14	0.58 ± 0.24
apoe−/−	0.40 ± 0.19	12.55 ± 1.40[a]	0.51 ± 0.01
APOE3/*apoe−/−*	0.43 ± 0.27	3.41 ± 0.23[a]	0.61 ± 0.12

[a] Significant difference from control values ($p < 0.05$).

palmitin containing spots were scraped and dissolved in 0.5 M acetic acid and assayed for radioactivity by scintillation counting.

Measurement of Nascent VLDL Particle Size—Hepatocytes were incubated for 24 h in SF-HF medium containing 0.75 mM oleic acid complexed to albumin. Medium from 6 wells (~6 × 10⁶ hepatocytes) was pooled, and VLDL was isolated by density gradient ultracentrifugation after addition of 0.35 g of KBr/ml medium. A salt solution of 1.0063 g/ml (containing 0.2 M NaCl and 270 μM Na-EDTA) was layered upon the medium, and centrifugation was performed for 24 h at 24.000 rpm and 4 °C in a TST41–14 rotor in a Centricon T-1080 ultracentrifuge (Milan, Italy). VLDL particles thus obtained were allowed to adhere to hydrophilic carbon films and immersed in 1% potassium phosphotungstate (pH 7.4) as a negative stain. Electron micrographs were obtained in a Philips CM100 electron microscope. Size distribution, based on measurement of at least 1600 particles per strain, was determined using Quantimet 520+ software (Leica, Cambridge, UK).

Miscellaneous—Protein concentrations were determined according to Lowry *et al.* (31) using bovine serum albumin (Sigma) as standard.

Statistical Analysis—Analyses of data from the three groups (APOE3/*apoe−/−*, *apoe−/−*, and control) were performed using a one-way analysis of variance, followed by the post-hoc Student Newman-Keuls test. Comparisons of data from two groups were performed using the Student's *t* test, when appropriate.

RESULTS

Characteristics of APOE3/apoe−/− Mice—To check distribution of APOE3 expression in the transgenic mice, total
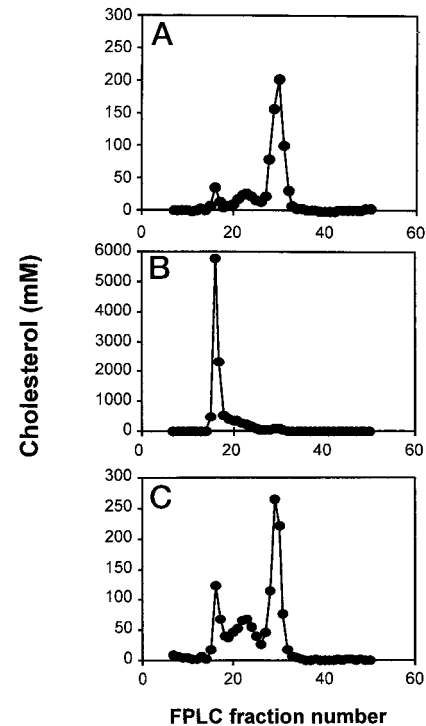

FIG. 3. **Cholesterol profile after fast protein liquid chromatography separation of plasma lipoproteins on a Superose 6B column.** Plasma of at least three animals/group was pooled, and 0.2 ml was applied to the column and eluted with phosphate-buffered saline at a flow rate of 0.5 ml/min. Cholesterol in the various fractions was measured enzymatically. *A*, control (C57BL/6J) mice. *B*, apoE-deficient mice. *C*, APOE3/*apoe−/−* mice. Note the difference in scale between the *y* axis of *B* and the *y* axes of *A* and *C*.

mRNA was isolated from various organs. Northern blot analysis showed that the APOE3/*apoe−/−* mice express human APOE3 in liver, lungs, and spleen. To a lesser extent, expression was also observed in brain, muscle, heart, and skin (Fig. 1). A similar expression pattern has been reported for endogenous *apoe* in mice (32, 33). Levels of human apoE3 in plasma were only 61.2 ± 5.3 μg/dl. For comparison, levels of endogenous apoE in C57BL/6 mice are 6.8 ± 0.2 mg/dl (34).

Immunoelectron microscopic studies were performed to determine the localization of apoE3 in livers of the transgenic mice. Particularly perivenous hepatocytes were strongly labeled at their microvilli lining the sinusoidal membranes (Fig. 2A). Multivesicular bodies contained apoE3 (Fig. 2B), in line with the role of apoE in remnant uptake. Association of apoE3 with budding Golgi and trans-Golgi structures was also observed, mainly with electron lucent material (Fig. 2C). Peroxisomes (Fig. 2D) were labeled in the characteristic, cluster-like fashion previously described for endogenous apoE in rat liver by Hamilton *et al.* (21).

Plasma Lipids—On regular low fat/low cholesterol lab chow, plasma cholesterol levels in APOE3/*apoe−/−* mice were slightly higher than those in controls but much lower than in *apoe−/−* mice (Table I). Plasma triglyceride and free fatty acid levels were similar across all groups (Table I). Separation of plasma lipoproteins using Superose 6B revealed that the characteristic elevation of cholesterol in the VLDL-sized lipoprotein fractions in *apoe−/−* mice was largely reversed by introduction of APOE3 (Fig. 3). Yet cholesterol levels were still elevated in the VLDL- and intermediate density lipoprotein/low density lipoprotein-sized fractions, suggesting that defective clearance of remnant particles in apoE-deficient mice is not completely restored by introduction of low levels of apoE3 (~1% of mouse

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TABLE II

Plasma concentrations of lathosterol, campesterol, and
β-sitosterol in chow-fed wild type (C57BL/6J),
apoe−/−, and APOE3/apoe−/− mice

Blood samples were collected by cardiac puncture from halothane-anaesthetized mice after a 4-h fast. Sterol concentrations were determined by capillary gas chromatography in EDTA plasma samples. Values represent the means ± S.D., $n = 3$ in all groups. Campesterol and β-sitosterol levels from APOE3/apoe−/− mice were not significantly different from controls because of the low number of mice.

	Lathosterol	Campesterol	β-Sitosterol
	μM	*μM*	*μM*
Wild type	ND[a]	21.6 ± 1.8	9.3 ± 0.5
apoe−/−	22.5 ± 10.5[b]	228.9 ± 44.33[b]	59.1 ± 21.0[b]
APOE3/apoe−/−	2.9 ± 0.6[c]	64.3 ± 5.0	16.4 ± 0.9

[a] ND, not detectable.
[b] apoe−/− is significantly different from both other groups.
[c] APOE3/apoe−/− is significantly different from controls ($p < 0.05$).

TABLE III

Hepatic triglyceride, cholesterol, and cholesteryl ester
concentrations in chow-fed wild type (C57BL/6J),
apoe−/−, and APOE3/apoe−/− mice

Livers were removed from halothane-anaesthetized mice after a 4-h fast, carefully cleaned and weighed, and immediately frozen in liquid nitrogen for later lipid analyses. Lipids were analyzed using commercially available enzymatic kits, as outlined under "Experimental Procedures." Values represent the means ± S.D., $n = 5$ in all groups.

	Triglycerides	Free cholesterol	Cholesteryl esters
	nmol/mg liver	*nmol/mg liver*	*nmol/mg liver*
Wild type	20.6 ± 6.2	4.2 ± 0.2	1.1 ± 0.1
apoe−/−	64.1 ± 22.8[a]	5.2 ± 0.6[a]	2.0 ± 1.0
APOE3/apoe−/−	12.9 ± 3.8	3.9 ± 0.3	1.0 ± 0.5

[a] Statistically significant difference from WT en APOE3/apoe−/− levels ($p < 0.05$).

apoE level). ApoE3 was present in all lipoprotein fractions (data not shown).

Plasma levels of the phytosterols campesterol and β-sitosterol were orders of magnitude higher in apoE-deficient mice compared with levels in control mice. Plasma concentrations of the cholesterol precursor lathosterol were also very high in apoE-deficient mice. In APOE3/apoe−/− mice, plasma concentrations of these sterols were dramatically reduced compared with apoE-deficient mice but still tended to be elevated compared with control mice (Table II).

Hepatic Lipids—Table III summarizes the contents of triglycerides, free cholesterol, and cholesteryl esters in livers of wild type, apoe−/−, and APOE3/apoe−/− animals. Although cholesteryl esters were unchanged, triglyceride and free cholesterol concentrations were elevated in apoE-deficient mice when compared with controls, as shown before (6, 13). It is evident that hepatic fat accumulation associated with apoE deficiency in the mouse is completely prevented by APOE3 expression. In fact, the hepatic triglyceride content even tended to be lower in the APOE3/apoe−/− animals than in the controls.

Livers of apoE-deficient mice show a very characteristic pattern of fat disposition in perivenous hepatocytes, *i.e.* in the cells surrounding the central vein (Fig. 4). As expected, this pattern was absent in the APOE3/apoe−/− animals. No abnormalities in livers of these mice could be detected by routine histological examination.

Hepatic VLDL Production—Hepatic VLDL-triglyceride production rate was measured *in vivo* after intravenous injection of Triton WR 1339 after an overnight fast (Table IV). The VLDL-triglyceride production rate was reduced from 108 ± 22 μmol/kg/h in wild type controls to 35 ± 7 μmol/kg/h in apoE-
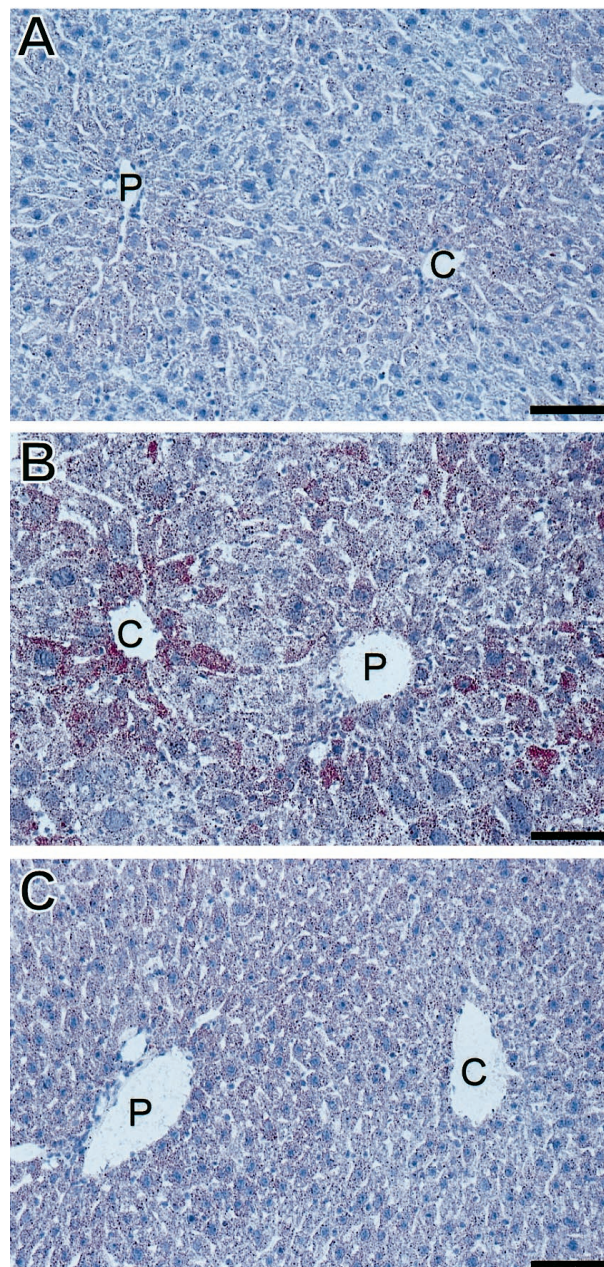

FIG. 4. **Oil-Red-O staining for neutral fat on frozen liver sections from wild type (*A*), apoe−/− (*B*), and APOE3/apoe−/− (*C*) mice reveals intense staining of hepatocytes surrounding the central vein in apoe−/− livers, indicative for the presence of fat in these perivenous cells.** In contrast, no specific localization of fat was noted in livers from wild type or APOE3/apoe−/− mice. *C*, central vein; *P*, portal vein. Original magnification, 50×. *Bar* indicates 0.2 mm.

deficient mice ($p < 0.005$). The production rate in APOE3/apoe−/− mice was highly comparable with that in control mice.

Hepatic production of apoB48 and apoB100 was determined in separate groups of mice according to Li *et al.* (26). The pool size of apoB100 and in particular of apoB48 was expanded in apoE-deficient mice. Introduction of APOE3 decreased apoB100 pool size to control values, as shown in Table IV. The pool size of apoB48 in APOE3/apoe−/− mice was larger than in controls. The production rate of B48 was similar among all groups, whereas the production rate of B100 was slightly but not significantly lower in apoE-deficient and APOE3/apoe−/− mice than in the controls.

TABLE IV

The in vivo VLDL-triglyceride and apoB production rates in chow-fed wild type (C57BL/6J), apoe−/−, and APOE3/apoe−/− mice

Hepatic VLDL-triglyceride production rates were calculated from the increases in plasma triglyceride concentration after intravenous injection of Triton WR 1339. For determination of apoB secretion, EDTA plasma was obtained from untreated mice and at 3 h after injection of Triton WR1339. VLDL was isolated by ultracentrifugation and subjected to SDS-polyacrylamide gel electrophoresis (4–15%), followed by Coomassie staining of the gel. Intensities of staining were measured with laser densitometry and quantified by comparison with human apoB standard. Values represent the means ± S.D. with $n \geq 4$ in all groups. TG, triglycerides; PR, production rate.

	TG PR	apoB pool size		apoB PR	
		B48	B100	B48	B100
	$\mu mol/kg/h$	μg	μg	$\mu g/h$	$\mu g/h$
Wild type	108 ± 22	5.2 ± 1.8	8.9 ± 1.9	30.2 ± 4.0	26.6 ± 7.3
apoe−/−	35 ± 7[a]	417 ± 103[a]	51.0 ± 20.5[a]	29.0 ± 17.9	15.6 ± 11.6
APOE3/*apoe−/−*	104 ± 27	38.4 ± 11.5[a]	11.3 ± 5.3	29.1 ± 22.1	15.8 ± 9.1

[a] Significant difference from wild type control.

To fully exclude potential interference of nonhepatocytic factors that may influence the VLDL production process *in vivo*, VLDL-triglyceride secretion was also studied in hepatocytes in primary culture using [3H]glycerol labeling. Fig. 5 shows the percentage of newly synthesized 3H-labeled triglyceride secreted into the medium by cells isolated from the three mouse strains. Secretion of VLDL-associated 3H-labeled triglyceride into the culture medium was clearly decreased for apoE-deficient cells when compared with control cells both in the absence and presence of oleate. In contrast, VLDL-3H-labeled triglyceride secretion by APOE3/*apoe−/−* cells was similar or even higher than that by control cells, indicating that the presence of APOE3 fully restores the capacity of VLDL-triglyceride secretion in apoE-deficient hepatocytes *in vitro*.

Fig. 6 shows that the average size of VLDL particles produced by cultured apoe−/− hepatocytes was less than that of particles produced by control cells and by APOE3/*apoe−/−* cells. In the latter case, the size distribution curve showed a clear skewing toward larger particle sizes when compared with controls. It should be noted that partial lipolysis of secreted VLDL particles during the 24-h incubation period may have occurred.

Effects of Adenovirus-mediated Introduction of APOE3 on in Vivo VLDL-triglyceride Production—To investigate to what extent hepatic VLDL-triglyceride production actually depends on APOE3 gene expression, APOE3 was introduced at different levels in apoE-deficient mice by liver-specific adenoviral transduction. Introduction of the APOE3 gene reduced cholesterol levels dramatically relative to LacZ-injected apoE-deficient mice when plasma apoE3 levels remained relatively low. However, both plasma cholesterol and triglyceride levels increased again when high levels of apoE3 in plasma were achieved (Table V), probably because of inhibition of lipoprotein lipolysis by excess apoE (2, 3, 35, 36). Five days after virus injection, the *in vivo* VLDL production was measured by the Triton WR1339 procedure. VLDL triglyceride production rates were stimulated up to 500% in mice injected with APOE3-containing virus compared with LacZ-injected controls (Table V). Hepatic VLDL-triglyceride production was not linearly related to hepatic APOE3 mRNA levels but showed a treshold value of about 0.7 arbitrary units (Fig. 7*A*). However, strong correlations were observed between the VLDL-triglyceride production rate on the one hand and the amount of apoE/mg VLDL-protein (Fig. 7*B*) or apoE/mg VLDL-triglycerides (Fig. 7*C*) on the other hand. This indicates that the relative amount of apoE per particle is a determinant of the VLDL-triglyceride production rate by the liver.

DISCUSSION

The results presented in this study are consistent with a regulatory role of apoE in hepatic VLDL-triglyceride production in the mouse, providing further evidence for a physiologi-
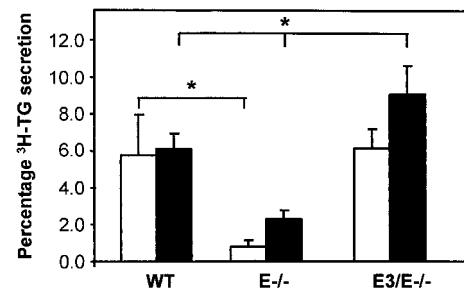

FIG. 5. **Percentage of newly synthesized 3H-labeled triglycerides secreted into medium by cultured hepatocytes from wild type (C57BL/6J), *apoe−/−*, and APOE3/*apoe−/−* mice, after incubation with [3H]glycerol for 3 h.** Lipids were extracted from media and cells, followed by separation by TLC. Triglyceride containing spots were scraped and dissolved in 0.5 M acetic acid and assayed for radioactivity by scintillation counting. *Black bars* represent incubations in which lipogenesis was stimulated with 0.75 mM oleate, and the *white bars* represent incubations in the absence of oleate. *, significant difference ($p < 0.05$).

cal function of this ubiquitous apolipoprotein in regulation of intracellular lipid metabolism in the liver. The transgenic mice used for these studies showed a relatively high expression of the transgene in the liver. Immunoelectron microscopical examination of livers of APOE3/*apoe−/−* mice revealed the presence of apoE3 at locations similar to those reported for endogenous apoE in rat liver (21). The apoprotein was found at the microvilli of the hepatocytic sinusoidal plasma membrane, consistent with its binding to heparan sulfate proteoglycans as well as to remnant lipoproteins (9, 10, 37). Inside the hepatocytes, apoE3 was also localized in putative Golgi secretory vesicles in association with electron lucent material possibly representing VLDL particles. This observation is consistent with the idea that apoE associates with VLDL prior to particle secretion, as also indicated by Hamilton *et al*. (38) and Fazio and Yao (39). Localization of apoE3 in peroxisomes is consistent with observations made by Hamilton *et al*. (21).

Plasma cholesterol levels of the APOE3/*apoe−/−* mice were strongly reduced when compared with levels in the apoE-deficient mice but still significantly higher than control values. The fast protein liquid chromatography analysis revealed that the (remnant) VLDL-cholesterol levels in APOE3/*apoe−/−* mice were still significantly higher than in control mice. Thus, it is likely that lipoprotein (remnant) uptake is not fully restored by introduction of APOE3 in the apoE-deficient mice. This is likely due to the fact that plasma levels of apoE3 remained much lower than those reported for endogenous apoE in C57Bl/6J mice (34). In addition, it has been shown that replacement of endogenous apoE by human apoE3 in mice by the knock-in approach causes elevated plasma cholesterol levels after a high fat diet (40), indicating that the human protein is less efficient in mediating lipoprotein uptake than the mouse protein is.

FIG. 6. **Effects of apoE on nascent VLDL-particle size distribution.** Hepatocytes were isolated and cultured for 24 h in 0.75 mM oleic acid-containing serum- and hormone-free medium. Media of ~6 × 10^6 cells were pooled, and VLDL was isolated by density gradient ultracentrifugation. Size distribution was determined after negative stain electron microscopy. *A*, control hepatocytes. *B*, *apoe*−/− hepatocytes. *C*, APOE3/*apoe*−/− hepatocytes. The differences between the three groups were significant ($p < 0.05$) as determined by one-way analysis of variance, followed by Student's Newman-Keuls test.
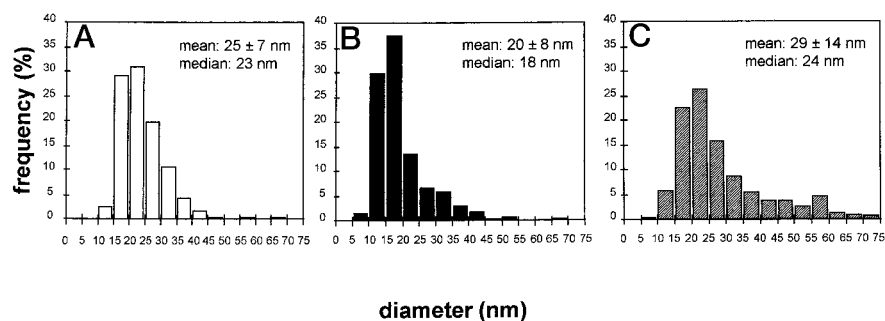

TABLE V

Triglyceride secretion rates in individual mice after adenovirus transduction with APOE3

Five days after virus transduction, Triton WR 1339 was injected, and blood samples were taken at regular intervals for determination of the triglyceride production rate (PR). For comparison, results from the transgenic APOE3/*apoe*−/− mouse are also given. AU, arbitrary units, compared to GAPDH mRNA expression; TG, triglycerides; CH, total cholesterol.

Gene	TG	CH	ApoE (plasma)	APOE3 mRNA in liver	PR	ApoE/mg VLDL protein
	mM	*mM*	*mg/dl*	*AU*	*μmol/kg/h*	*μg/mg*
Ad-LacZ	0.79 ± 0.22	20.47 ± 1.91			45.8 ± 8.4	
Ad-APOE3	0.48	0.90	2.13	0.68	34.3	2.92
	0.59	1.18	3.13	0.64	62.3	2.92
	0.44	1.30	1.47	0.41	47.73	2.92
	0.82	1.66	50.50	1.03	57.63	22.94
	1.43	3.31	69.00	1.35	365.5	48.45
	2.89	4.53	101.26	0.98	427.7	82.58
	1.43	4.01	141.12	1.12	196.2	25.49
	4.73	4.37	103.31	1.22	653.2	86.61
	5.55	7.80	161.85	1.52	344.7	33.87
APOE3/*apoe*−/−	0.43 ± 0.27	3.41 ± 0.23	0.06 ± 0.004	0.25 ± 0.05	104 ± 27	ND[a]

[a] ND, not determined.

Defective clearance of apoE3-containing lipoproteins is further supported by our finding that plasma levels of the plant sterols campesterol and β-sitosterol remain elevated in APOE3/*apoe*−/− mice in comparison with wild type controls. Because these plant sterols are derived from diet and because sterol absorption is not affected in apoE-deficient mice (41), their elevated plasma concentrations must reflect impaired clearance of chylomicron remnants. This finding demonstrates that plasma levels of these sterols are not primarily determined by their absorption efficiency from the intestine and questions the validity of their use as indicators of intestinal cholesterol absorption, as proposed by Miettinen and co-workers (42, 43).

Introduction of APOE3 in apoE-deficient mice resulted in reversal of fat accumulation in the liver and in an almost 3-fold increase in VLDL-triglyceride production, comparable with control values, *in vivo* as well as *in vitro* in primary hepatocytes in culture. To determine whether the impaired VLDL-triglyceride secretion *in vivo* in apoE-deficient mice is due to secretion of a reduced number of VLDL particles or to a reduced amount of triglyceride per particle, hepatic apoB secretion rates were measured in the *in vivo* situation. The secretion rate of apoB48 by the liver was not influenced by apoE status. The secretion rate of apoB100 appeared to be somewhat decreased both in apoE-deficient and in APOE3/*apoe*−/− mice, but the differences did not reach statistical significance because of the large variation in results (Table IV). Together, therefore, data indicate that apoE deficiency leads to impaired packaging of triglycerides into VLDL particles rather than to secretion of a reduced number of VLDL particles. This is consistent with the observation that the average size of VLDL particles produced by apoE-deficient hepatocytes cultured in the presence of oleate were smaller than those from control and APOE3/*apoe*−/− mouse hepatocytes (6). In fact, introduction of APOE3 resulted in formation of a considerable number of relatively large particles,

as is evident from the size distribution diagram shown in Fig. 6.

To determine whether apoE actually regulates a rate-determining step in the VLDL-triglyceride production cascade, the APOE3 gene was introduced at different levels in livers of apoE-deficient mice by adenoviral transduction. The plasma cholesterol concentrations dropped dramatically, even at low levels of expression of the APOE3 gene. Yet secretion of VLDL-triglycerides was still impaired under these conditions, delineating the differential functions of apoE in lipoprotein uptake and secretion, respectively. Secretion of VLDL-triglyceride was increased only after reaching a certain treshold of APOE3 mRNA in the liver. We found a strong positive correlation between VLDL-triglyceride production rate and the amount of apoE per particle, expressed either as mg of VLDL-protein or as mg of VLDL-triglyceride. Theoretically, it is possible that VLDL-triglyceride secretion drives apoE3 secretion because a larger particle, corresponding to a higher VLDL-triglyceride secretion, obviously can contain more apoE3 than a smaller particle can. However, because of the fact that differential expression of APOE3 is the only variable between these mice, we propose that intracellular association of apoE3 molecules with nascent VLDL particles results in formation of larger particles.

Comparison of data obtained with virus-injected mice and APOE3/*apoe*−/− mice shows that there is a clear difference in the effect of low APOE expression in the transgenic mice as compared with low dose Ad-APOE injection in *apoe*−/− mice. This is likely due to the fact that in the transgenic mice all liver cells express APOE, whereas in the low dose Ad-APOE-injected animals only a relatively small fraction of liver cells may express APOE. At a high dose of Ad-APOE, probably resulting in APOE expression in more liver cells, it is apparent that the hepatic triglyceride production rate is severalfold increased as compared with *apoe*−/− mice and with the transgenic APOE3/

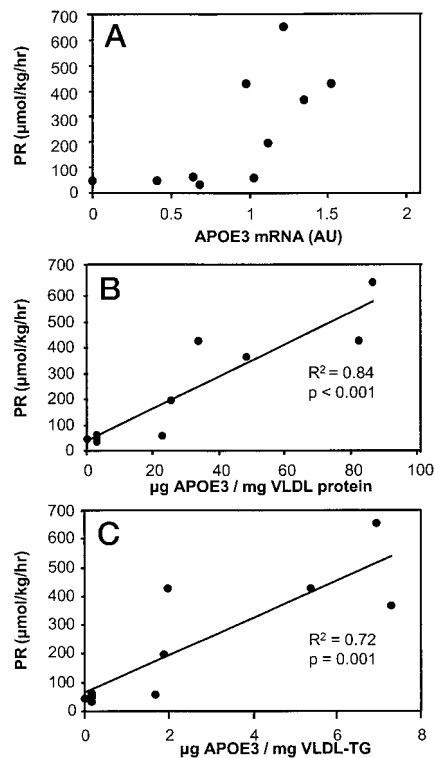FIG. 7. **Correlations between VLDL-triglyceride production rate and APOE3 expression.** ApoE-deficient mice were injected with variable amounts of adenovirus containing APOE3 cDNA (Ad-APOE3). VLDL-triglyceride production rates were measured using the Triton WR 1339 method. ApoE3 concentrations were determined using enzyme-linked immunosorbent assay in total plasma and in VLDL isolated by ultracentrifugation. *A*, relationship between VLDL-triglyceride production rate and hepatic APOE3 mRNA levels ($R^2 = 0.49$). *B*, relationship between VLDL-triglyceride production rate and the concentration of apoE3/mg VLDL-protein. *C*, relationship between VLDL-triglyceride production rate and the amount of apoE3/mg VLDL-triglyceride.

$apoe-/-$ mice (Table V), indicative for a direct APOE-mediated effect on this process. Thus, in our view, in mice given a low dose of Ad-APOE, the small fraction of liver cells that does express APOE will have a normalized or increased triglyceride production rate, but this is not sufficient to stimulate the triglyceride production rate of the liver as a whole.

Results of these studies indicate that apoE serves a distinct role in regulation of intrahepatic lipid metabolism related to the VLDL production process. The finding that overexpression of APOE3 in $apoe-/-$ livers by adenoviral transfection actually stimulates hepatic VLDL-triglyceride secretion implies that the apolipoprotein actually controls an important step in the particle assembly cascade. This view is supported by recently published studies using different experimental set-up (44, 45). Firstly, Willems van Dijk *et al.* (44) demonstrated that adenovirus-mediated APOE gene transfer increased hepatic VLDL-triglyceride production in normal C57BL/6 mice at 5 days after virus injection. Secondly, Huang *et al.* (45) reported a 50% increase in hepatic VLDL-triglyceride production in human APOE3 expressing mice and increased VLDL-triglyceride production in McA-RH7777 cells overexpressing human APOE2, E3 or E4. Because hepatic apoB secretion is not influenced by apoE deficiency or APOE3 expression, the role of apoE must be related to lipid packaging during the formation of VLDL particles, which is supported by the apoE dependence of VLDL particle size distribution. Because overproduction of large VLDL particles by the liver is an important contributor to the development of hyperlipidemia in humans, an important independent risk factor for development of atherosclerosis, this finding may have important implications for our understanding of the etiology and, potentially, for treatment of hyperlipidemia.

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Apolipoprotein E Participates in the Regulation of Very Low Density Lipoprotein-Triglyceride Secretion by the Liver

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