Impaired Secretion of Very Low Density Lipoprotein–Triglycerides by Apolipoprotein E–deficient Mouse Hepatocytes

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Abstract

To explore mechanisms underlying triglyceride (TG) accumulation in livers of chow-fed apo E–deficient mice (Kuipers, F., J.M. van Ree, M.H. Hofker, H. Wolters, G. In't Veld, R.J. Vonk, H.M.G. Princen, and L.M. Havekes. 1996. *Hepatology.* 24:241–247), we investigated the effects of apo E deficiency on secretion of VLDL-associated TG (*a*) in vivo in mice, (*b*) in isolated perfused mouse livers, and (*c*) in cultured mouse hepatocytes. (*a*) Hepatic VLDL-TG production rate in vivo, determined after Triton WR1339 injection, was reduced by 46% in apo E–deficient mice compared with controls. To eliminate the possibility that impaired VLDL secretion is caused by aspecific changes in hepatic function due to hypercholesterolemia, VLDL-TG production rates were also measured in apo E–deficient mice after transplantation of wild-type mouse bone marrow. Bone marrow–transplanted apo E–deficient mice, which do not express apo E in hepatocytes, showed normalized plasma cholesterol levels, but VLDL-TG production was reduced by 59%. (*b*) VLDL-TG production by isolated perfused livers from apo E–deficient mice was 50% lower than production by livers from control mice. Lipid composition of nascent VLDL particles isolated from the perfusate was similar for both groups. (*c*) Mass VLDL-TG secretion by cultured apo E–deficient hepatocytes was reduced by 23% compared with control values in serum-free medium, and by 61% in the presence of oleate in medium (0.75 mM) to stimulate lipogenesis. Electron microscopic evaluation revealed a smaller average size for VLDL particles produced by apo E–deficient cells compared with control cells in the presence of oleate (38 and 49 nm, respectively). In short-term labeling studies, apo E–deficient and control cells showed a similar time-dependent accumulation of [^{3}H]TG formed from [^{3}H]glycerol, yet secretion of newly synthesized VLDL-associated [^{3}H]TG by apo E–deficient cells was reduced by 60 and 73% in the absence and presence of oleate, respectively. We conclude that apo E, in addition to its role in lipoprotein clearance, has a physiological function in the VLDL assembly–secretion cascade. (*J. Clin. Invest.* 1997. 100:2915–2922.) Key words: transgenic mice • lipoproteins • liver • cholesterol • apolipoprotein B

Introduction

Apolipoprotein E (apo E) is an important protein constituent of triglyceride (TG)[1]-rich chylomicrons and VLDL. apo E functions as a ligand in the receptor-mediated uptake of these lipoproteins by the liver (1) and may modulate their lipoprotein lipase–mediated processing (2, 3). It has also been suggested that apo E plays a role in the secretion–recapture process (4, 5). In this process, internalization of circulating lipoproteins is facilitated through binding of the particles to heparan sulfate proteoglycans and subsequent enrichment with apo E in the hepatic sinusoidal space of Disse. Furthermore, recent in vitro studies have indicated that apo E may serve a function in intracellular metabolism and distribution of endocytosed lipids in macrophages and hepatoma cells (6, 7).

The generation of apo E–deficient mice has established directly the importance of apo E in control of plasma cholesterol levels (8, 9). apo E–deficient mice show markedly elevated plasma cholesterol levels when fed a normal chow diet, due to accumulation of VLDL/LDL-sized particles in their circulation (10–12). These particles contain predominantly apo B48 and are relatively enriched in cholesterol and cholesterol ester and depleted in TG (10–12). As recently reviewed by Plump and Breslow (8), these particles are mainly remnants of intestinal chylomicrons.

Data in the literature suggest that apo E may also have a function in the assembly and/or secretion of VLDL by the liver. Studies by Strobl et al. (13) showed that stimulation of hepatic VLDL production in rats by feeding them sucrose-rich diets enhances apo E synthesis and secretion by promoting transcription of the apo E gene. Hamilton et al. (14) demonstrated that apo E is present in nascent VLDL particles within putatively forming Golgi secretory vesicles of rat hepatocytes. Studies by Fazio et al. (15) showed that most of the apo E secreted by human hepatoma cells (HepG2) becomes associated with large TG-containing lipoproteins when lipogenesis and TG secretion are stimulated, whereas the total amount of apo E synthesized and secreted is not affected. More recently,

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1. *Abbreviations used in this paper:* LDH, lactate dehydrogenase; LFC, low fat/low cholesterol; TG, triglyceride.

Fazio and Yao (16) reported that the association between apo E and TG-rich lipoproteins in HepG2 and rat hepatoma cells (McA-RH7777) takes place intracellularly rather than extracellularly, after their secretion into the medium. Finally, recent studies by our group demonstrated that apo E–deficient mice accumulate large amounts of TG in their livers (17). Therefore, altogether, scattered evidence is available to suggest that apo E has a function in VLDL assembly and/or secretion.

In this paper, we investigated the effects of apo E deficiency on VLDL-TG synthesis and secretion in vivo as well as in vitro, using apo E–deficient mice, perfused apo E–deficient mouse livers, and isolated apo E–deficient hepatocytes. These studies demonstrate unequivocally that VLDL-TG production is severely impaired in the absence of apo E, whereas TG synthesis in apo E–deficient hepatocytes is not affected. In addition, impaired VLDL-TG production is also observed in apo E–deficient mice in which plasma cholesterol levels have been normalized by transplantation of wild-type bone marrow. These data lead us to conclude that apo E has a modulating role in the VLDL secretion cascade, representing a novel physiological function of this ubiquitous apolipoprotein.

Methods

Animals. apo E–deficient mice were generated as described previously (12). apo E–deficient and control (C57BL/6J) mice were housed in a light- and temperature-controlled environment and had free access to water and food.

To induce bone marrow aplasia, apo E–deficient and control mice (age 5–6 wk) were exposed to a single dose of 13 Gy (0.28 Gy/min, 200 kV, 4 mA) x-ray total body irradiation using an Andrex Smart 225 (Andrex Radiation Products A/S, Copenhagen, Denmark) with a 4-mm aluminum filter, 1 d before transplantation. Bone marrow cell suspensions were isolated by flushing the femurs and tibias from control mice with PBS. Single-cell suspensions were prepared by passing the cells through a 30-μm nylon gauze. Irradiated apo E–deficient and control recipients received 1.5×10^7 bone marrow cells by intravenous injection into the tail vein. Mice used for bone marrow transplantation experiments were housed in sterilized filter-top cages and fed sterilized chow (SRM-A; Hope Farms BV, Woerden, The Netherlands) containing 5.7% fat (wt/wt). Drinking water was supplied with antibiotics (83 mg/liter ciprofloxacin and 67 mg/liter polymyxin B sulphate) and 6.5 g/liter glucose.

For liver perfusion studies, female apo E–deficient and control mice were used that were fed a low fat/low cholesterol (LFC) semisynthetic diet, composed essentially according to Nishina et al. (18) (Hope Farms BV). The LFC diet contained 50.5% sucrose, 12.2% corn starch, 5% corn oil, and 5% cellulose, by weight, and was fed to the mice for a period of 3 wk.

Male apo E–deficient and control mice were used for cell isolations. These mice were fed a normal chow diet (RMH-B; Hope Farms BV) containing 6.2% fat and ~ 0.01% cholesterol by weight.

Experimental protocols were approved by the Ethics Committee for Animal Experiments of the University of Groningen.

Serum lipid and lipoprotein analysis. Levels of total plasma cholesterol and TG were measured, with or without free glycerol, using commercially available enzymatic kits (236691 and 701904; Boehringer Mannheim GmbH, Mannheim, Germany, and 337-B; Sigma Chemical Co., St. Louis, MO, respectively). The phospholipid content of nascent VLDL particles secreted from the perfused mouse liver was determined using an analytical kit (Wako Chemicals GmbH., Neuss, Germany). Total cholesterol and free cholesterol contents in nascent VLDL fractions were measured fluorometrically (excitation: 325 nm; emission: 415 nm) in a phosphate buffer (pH = 7.4) using cholesterol oxidase and peroxidase (Boehringer Mannheim GmbH), 0.5% Triton X-100 (Merck, Darmstadt, Germany), 20 mM cholic acid, and 4 mg/dl para-hydroxy-phenyl-acetic acid (Sigma Chemical Co.). Before measurements, total cholesterol was extracted using KOH/ethanol/hexane, and free cholesterol using ethanol/hexane. Esterified cholesterol was calculated as the difference between total and unesterified cholesterol.

Protein concentrations in VLDL fractions were measured according to Lowry et al. (19) using BSA (Sigma Chemical Co.) as standard.

In vivo hepatic VLDL-TG production using Triton WR1339. Untreated and bone marrow–transplanted mice were injected intravenously with 500 mg of Triton WR1339 (Sigma Chemical Co.) per kg body wt as a 15-g/dl solution in 0.9% NaCl after an overnight fast. Previous studies have shown that plasma VLDL clearance is virtually completely inhibited under these conditions (20). Blood samples (50 μl) were taken at 0, 1, 2, 3, and 4 h after Triton WR1339 injection, and plasma TG was measured enzymatically as described above. Plasma TG concentrations were related to the body mass of the animals, and the hepatic TG production rate was calculated from the slope of the curve and expressed as micromoles per hour per kilogram body weight.

Liver perfusion experiments. Experiments were always begun between 10 and 11 a.m. Fed mice were anesthetized by intraperitoneal injection of 0.5 ml/kg Hypnorm (Janssen-Cilag Pharmaceutica BV, Tilburg, The Netherlands) and 12.5 mg/kg midazolam (Roche Netherlands BV, Mijdrecht, The Netherlands). After cannulation of the portal vein with an Abbocath-T cannula (26 G × 19 mm; Abbott Laboratories, Kent, UK), livers were isolated and perfused (1.5 ml/min) in a recycling fashion. The liver and the perfusate were maintained at 37°C throughout the experiment. The recirculating perfusate (20–30 ml) was an RPMI buffer (RPMI 1640 medium; ICN Biomedicals, Inc., Costa Mesa, CA), pH 7.5, containing 1 mM vitamin C, 5 mM glutathione, and 2.5% (wt/wt) BSA. Oleate was added as a BSA-complex to the perfusate to reach a concentration of 1 mM in all experiments. The perfusate was gassed with 95% O_2/5% CO_2. All the perfused livers were functionally viable by gross appearance after a 3-h perfusion period. At termination of the experiment, livers were perfused with blue dye (Polysciences Inc., Warington, PA) to assess grade of perfusion, cleansed of all extrahepatic nonperfused tissue, and weighed.

Before isolation of nascent VLDL, the 20–30 ml of perfusate was concentrated to 10 ml (Centriprep 30; Amicon, Inc., Danvers, MA). Nascent VLDL fractions ($d < 1.006$ g/ml) were isolated by ultracentrifugation at 40,000 rpm in a swingout rotor (model SW-40; Beckman Instruments International S.A., Geneva, Switzerland) for 18 h at 5°C. Each VLDL sample was dialyzed extensively against PBS overnight at 4°C. The lipid composition of the nascent VLDL particles was analyzed as described above.

Preparation and culture of mouse hepatocytes. Hepatocytes were isolated from mouse livers as described previously by Klaunig et al. (21). Briefly, the portal vein was cannulated with a 22-gauge plastic cannula. First, the liver was perfused with calcium-free HBSS containing glucose (10 mM) and Hepes (10 mM), pregassed with 95% O_2/5% CO_2, and adjusted to pH 7.42, at a flow rate of 4 ml/min. Subsequently, the liver was perfused with a collagenase solution (20 mg collagenase [Sigma Chemical Co.]/200 ml calcium-containing [5 mM] HBSS) for ~ 10–15 min until swelling of the liver was observed. Hepatocytes were then gently released from the surrounding capsule. Isolated hepatocytes were washed three times with Williams' E medium (Life Technologies Ltd., Paisley, UK) containing 5% (wt/wt) BSA. Hepatocytes were isolated with similar yields from livers of control and apo E–deficient mice, with average values of $\sim 6 \times 10^7$ cells/liver. The viability of hepatocytes was assessed by trypan blue (final concentration 0.2%) exclusion immediately after isolation, and varied between 65 and 80% of the total amount of cells isolated. No differences were observed in this respect between cells isolated from apo E–deficient and control mice. 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 viable cells/well in 2 ml culture medium. After a 2-h adherence period, nonviable cells were removed from the cultures by careful washing. The culture medium consisted of Williams' E medium supplemented with 10% FCS (Bio Withaker, Verviers, Belgium), 100 μg/ml of penicillin and streptomycin (Life Technologies Ltd.), 4 mU/ml insulin, and 0.02 μg/ml dexamethasone (both from Novo Nordisk Pharma BV, Amsterdam, The Netherlands) as described previously by Princen et al. (22). Cells were incubated at 37°C in an atmosphere of 95% air/5% CO_2 and rapidly formed monolayers.

The total synthesis and secretion of proteins by cultured hepatocytes was determined by [^{3}H]leucine incorporation into TCA-precipitable proteins. For this purpose, [^{3}H]leucine (2 μCi/ml per well; Amersham International, Little Chalfont, UK) was added to the medium 3 h before termination of cell incubation. Both the protein synthesis and the secretion of newly synthesized protein were similar in overnight cultured hepatocytes isolated from apo E–deficient and control mice.

Lactate dehydrogenase (LDH) activity in media and cells was determined as reported previously (23). No differences were observed in LDH leakage from hepatocytes isolated from apo E–deficient or control mice. In all cases, > 95% of the LDH activity was found intracellularly.

Experimental protocols for cultured hepatocytes. After an overnight culture, FCS-containing medium was removed from the wells, and cells were washed twice with FCS/hormone-free medium. Cells were incubated subsequently with FCS/hormone-free medium (1 ml/well, pH 7.4) containing 0.25 mM BSA only, or BSA–oleate complex (0.75 mM oleate/0.25 mM BSA) at 37°C for either 4 or 24 h. In all experiments, oleate was used to stimulate hepatocytic lipogenesis. Medium containing BSA–oleate complexes was freshly prepared on the day of each experiment.

To determine the glycerolipid synthesis and secretion rates, [^{3}H]glycerol (4.4 μCi, 25 μM final concentration; Amersham International) or [^{3}H]oleate (2 μCi, in 10 μl ethanol; Amersham International) was added to each well according to different experimental protocols (see figure citations in Results). All incubations were performed in triplicate and terminated by placing the culture plates on ice. Media were collected and centrifuged at 10,000 rpm for 2 min to remove suspended cells. Cells were washed three times with cold PBS and harvested using a rubber policeman in 2 ml PBS. Samples were frozen at −20°C before analysis.

Cholesterol synthesis by cultured cells was estimated by measuring [^{14}C]acetate (Amersham International) incorporation into sterols. Previous studies have indicated that this method yields relative values for cellular lipid synthesis similar to those obtained with [^{3}H]water in cultured hepatocytes (24).

Assessment of cellular and VLDL lipid content. Nascent VLDL particles ($d < 1.006$ g/ml) were isolated from the medium by ultracentrifugation at 41,000 rpm in a TST 41.14 rotor (Kontron Instruments, Milan, Italy) for 24 h at 4°C. The radiolabeled lipids from VLDL and cells were extracted with chloroform/methanol (1:2, vol/vol). Before lipid extractions, cells were first thawed and resuspended by passing through 26 G (0.45 × 25 mm) needles five times. Radiolabeled cellular TG, free cholesterol, cholesterol ester, and phospholipids were separated by TLC on silica gel 60 plates (Merck) with hexane/diethylether/acetic acid (80:20:1, vol/vol/vol) as resolving solution. Before TLC analysis, tripalmitin (Sigma Chemical Co.) in chloroform was added to each sample as a carrier for TG determination. After iodine staining of the silica gel plates, the spots containing the lipids of interest were scraped into vials and assayed for radioactivity by scintillation counting.

In another set of experiments, TG content of VLDL and cells was measured using a commercially available kit from Boehringer Mannheim GmbH as described above. Free cholesterol and cholesterol ester contents were determined according to the methods developed by Gamble et al. (25). Phospholipids were determined by measuring the phosphorus content of lipid extracts after perchloric acid treatment (26).

Electron microscopy. VLDL fractions were prepared for electron microscopy analysis within 1 h after their isolation by ultracentrifugation. The particles were allowed to adhere to hydrophilic carbon films and were immersed in 2% potassium phosphotungstate (pH 7.4) as a negative stain. Electron micrographs were obtained in a transmission electron microscope (model EM208; Philips Electron Optics, Eindhoven, The Netherlands). Size distribution of VLDL particles was determined using Quantimet 520+ software (Leica Cambridge Ltd., Cambridge, UK).

Western blotting of apo B. VLDL particles were isolated from the media by ultracentrifugation as described above and adsorbed to hydrated colloidal fumed silica (Cab-O-Sil, particle size 0.011 μm; Sigma Chemical Co.) as reported previously (27). The VLDL apo B and apo E contents were analyzed by immunoblotting as described by Lin et al. (28) using rabbit anti–rat apo B serum showing cross-reactivity with murine apo B (a kind gift of Roger A. Davis, San Diego State University, San Diego, CA) and rabbit anti–mouse apo E serum (kindly provided by Pieter H.E. Groot, SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, UK).

Results

In vivo VLDL-TG production rate in apo E–deficient mice: effect of bone marrow transplantation. The effect of apo E deficiency on the in vivo VLDL-TG production rate was determined by injecting apo E–deficient and control mice with Triton WR1339. Table I shows that the hepatic TG production rate was reduced in apo E–deficient mice compared with controls (61.1±3.6 vs. 112.6±19.4 μmol TG/h/kg, respectively). To verify whether this effect is attributable to the lack of apo E within the hepatocytes, and not to aspecific changes in liver function due to the hypercholesterolemia associated with apo E deficiency, wild-type mouse bone marrow cells expressing apo E were transplanted into apo E–deficient mice. In these mice, bone marrow–derived macrophages are the only sources of apo E, i.e., there is no expression of apo E in hepatocytes.

Table I. Plasma Lipid Levels and In Vivo VLDL-TG Production Rates in apo E–deficient and Control Mice: Effects of Bone Marrow Transplantation

Mice	*n*	TC	TG	PR
		mmol/liter	*mmol/liter*	*μmol TG/h/kg*
Control	3	1.8±0.2	2.8±0.2	112.6±19.4
apo E–deficient	3	18.1±1.4*	3.7±0.6	61.1±3.6*
Control Tx	3	1.5±0.2	2.9±0.3	109.8±23.3
apo E–deficient Tx	3	2.5±0.7	3.0±0.1	45.2±14.2*

Total cholesterol (*TC*) and TG levels were measured in the plasma of fasted apo E–deficient and control (C57BL/6J) mice using enzymatic kits (236691 and 701904, respectively; Boehringer Mannheim). Irradiated apo E–deficient and control mice were given transplants (*Tx*) of wild-type bone marrow cells as described in Methods. Hepatic VLDL-TG production rate (*PR*) was measured by injecting mice with Triton WR1339 (500 mg/kg). For the transplanted mice, Triton WR1339 was injected 16–20 wk after bone marrow transplantation. Plasma TG levels were determined before injection (0 min) and at 30, 60, 120, 180, and 240 min after Triton injection. Hepatic VLDL-TG production rate was calculated from the slope of the curve. *$P < 0.05$, indicating significant differences between untreated control and apo E–deficient mice, or between transplanted control and apo E–deficient mice, using Student's *t* test.

Bone marrow transplantation effectively reversed hypercholesterolemia in apo E–deficient mice (Table I), as reported previously by other groups (29, 30). Introduction of wild-type bone marrow into control mice had no effect on plasma lipid concentrations (Table I). In spite of normalized plasma lipid levels, VLDL-TG production rates remained inhibited in transplanted apo E–deficient mice compared with the transplanted control mice (45.2±14.2 vs. 109.8±23.3 μmol TG/h/kg, respectively) (Table I). These results indicate that the reduced hepatic TG production observed in apo E–deficient mice is related to the absence of apo E in the liver.

Secretion of nascent VLDL by isolated perfused mouse livers. To investigate the effects of apo E deficiency on nascent VLDL-TG production by polarized hepatocytes in their physiological environment, we performed studies with isolated perfused livers from apo E–deficient and control mice. To stimulate VLDL-TG production (18), mice were fed the sucrose-containing LFC diet for a period of 3 wk before the liver perfusion experiments.

VLDL-TG secretion by isolated perfused mouse livers was linear during a 3-h period of perfusion (data not shown), indicating that the liver remains functionally viable during this time period. VLDL-TG production by perfused livers from apo E–deficient mice was reduced by 50% compared with perfused control livers (Table II). In addition, the nascent VLDL particles produced by apo E–deficient livers had a lipid composition similar to nascent VLDL secreted by control livers (Table II).

Characteristics of nascent VLDL particles produced by mouse hepatocytes in primary culture. The TG and cholesterol contents of apo E–deficient cells cultured for 24 h in the absence of oleate (0.75 mM) were elevated significantly compared with control cells, whereas no differences in cholesterol ester and phospholipid content were found (Table III). The presence of oleate in the incubation medium resulted in a marked TG accumulation in both apo E–deficient and control cells compared with oleate-free conditions (Table III).

The secretion of nascent VLDL by cultured hepatocytes was quantitated through lipid content analysis of the < 1.006 g/ml fractions obtained by density gradient ultracentrifugation of media collected after 24 h of incubation. As shown in Fig. 1 (*left*), apo E–deficient cells produce slightly but significantly less VLDL-TG compared with control cells in the absence of oleate (77% of control values). The total mass lipid secretion by control cells was stimulated in the presence of oleate (compare *left* and *right panels* in Fig. 1). In contrast, VLDL-TG production by apo E–deficient cells was strongly reduced in the presence of oleate (39% of control values) (Fig. 1, *right*). Furthermore, the relative lipid composition of VLDL secreted by apo E–deficient and control cells appeared to be similar under all conditions (Fig. 1).

Western blot analysis revealed that apo B48 is the major form of apo B secreted by control and apo E–deficient mouse hepatocytes, which is consistent with the high level of apo B mRNA editing activity in mouse liver (31) (results not shown). As expected, apo E could be detected in VLDL particles produced by control cells but not in those from apo E–deficient cells (data not shown).

The size of the particles isolated from the < 1.006 g/ml fractions was determined by electron microscopy. As shown in Fig. 2 (*left*), no differences in size were found between apo E–deficient and control particles when cells were cultured under oleate-free conditions (41.8±0.7 vs. 43.8±2.0 nm, respectively). In contrast, when cells were cultured in the presence of oleate, the average diameter of apo E–deficient VLDL particles was smaller than that of control particles (38.3±3.3 nm vs. 49.5±1.9 nm, respectively) (Fig. 2, *right*). The average size of VLDL particles formed in the presence of oleate was significantly larger for control cells compared with oleate-free conditions (compare *left* and *right panels* in Fig. 2).

Since the size of apo E–deficient VLDL was smaller than control VLDL under oleate-stimulated conditions, it was investigated whether these particles could be recovered in higher density fractions upon ultracentrifugation. Therefore, we performed cell incubation experiments in the presence of 0.75 mM oleate and [^{3}H]glycerol to label the newly synthesized lipids. After 24 h of incubation, the medium was harvested, and VLDL was isolated by ultracentrifugation. Although the total amount of radiolabeled lipids secreted by apo E–deficient cells is decreased compared with control cells, most of the radioac-

Table II. VLDL-TG Production Rate and Relative Lipid Composition of Nascent VLDL Secreted by Perfused Livers from apo E–deficient and Control Mice

Nascent VLDL	VLDL-TG production	TG	FC	CE	PL
	μg TG/g liver	*% of total lipid (by wt)*			
Control	317±46	80±4	2±1	5±1	13±4
apo E–deficient	158±23*	75±6	2±1	9±5	17±5

Livers of female apo E–deficient and control (C57BL/6J) mice fed a sucrose-based semisynthetic (LFC) diet for 3 wk were perfused in a recirculating fashion as described in Methods. After 3 h of liver perfusion, perfusates were collected, and nascent VLDL was isolated by ultracentrifugation and analyzed for its lipid composition. TG, free cholesterol (*FC*), cholesterol ester (*CE*), and phospholipid (PL) contents of nascent VLDL particles were determined as described in Methods. Values are given as mean±SD of four individual liver perfusion experiments per group. *$P < 0.05$, indicating a significant difference between apo E–deficient and control perfused liver preparations, using Student's *t* test.

Table III. Cellular Lipid Content of apo E–deficient and Control Mouse Hepatocytes in Primary Culture

		TG	FC	CE	PL
		nmol/mg protein			
No oleate	Control	81±9	34±3	4±1	206±25
	apo E–deficient	142±50*	58±8*	5±1	251±44
With oleate	Control	277±79‡	39±6	6±2	255±52
	apo E–deficient	304±71‡	51±4*	9±2	286±46

Cells were cultured for 24 h in the absence or presence of oleate (0.75 mM) and were subsequently washed and harvested for lipid analysis. Cellular TG, free cholesterol (*FC*), cholesterol ester (*CE*), and phospholipid (*PL*) contents were measured as described in Methods. Values are expressed as mean±SD of four individual hepatocyte preparations per group. *$P < 0.05$, indicating a significant difference between apo E–deficient and control preparations under the same culture conditions. ‡$P < 0.05$, indicating a significant difference between hepatocyte preparations in the absence or presence of oleate, using Student's *t* test.

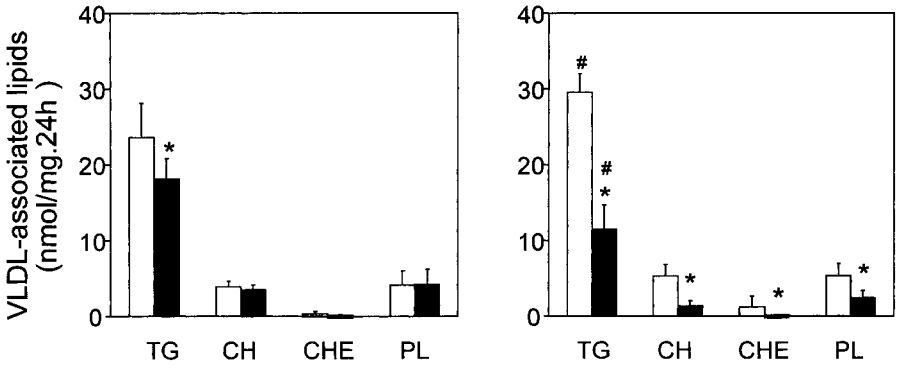

Figure 1. VLDL-associated lipid secretion by apo E–deficient and control mouse hepatocytes. Cells were cultured for 24 h in the absence (*left panel*) or presence (*right panel*) of 0.75 mM oleate as described in Methods. Lipoproteins were isolated from the < 1,006 g/ml fraction after density gradient ultracentrifugation of medium samples, and lipid composition was analyzed, as described in Methods. Values are expressed as mean±SD of four independent experiments in each group. $^{*}P < 0.05$, indicating significant differences between apo E–deficient (*black bars*) and control cells (*white bars*) under the same culture conditions. $^{\#}P < 0.05$, indicating significant differences between absence and presence of oleate, using Student's *t* test. *CH*, Cholesterol. *CHE*, Cholesterol ester. *PL*, phospholipid.

tivity is recovered from the < 1.006 g/ml fractions for both control and apo E–deficient cells (Fig. 3). Thus, these results indicate that the decreased recovery of apo E–deficient VLDL in the < 1.006 g/ml fraction is due to an impaired VLDL secretion by apo E–deficient hepatocytes, rather than to a shift in the density of apo E–deficient particles.

Secretion of newly synthesized TG by hepatocytes in primary culture. To compare the cellular synthesis and secretion of newly formed lipids by control and apo E–deficient cells, we performed incubation experiments with either [^{3}H]glycerol or [^{3}H]oleate to label the newly synthesized TG. Fig. 4 shows a similar time-dependent cellular accumulation of [^{3}H]TG formed from radiolabeled glycerol in apo E–deficient and wild-type cells, both in the absence (Fig. 4 *A*) and presence (Fig. 4 *B*) of oleate. Similar results were obtained in three additional independent experiments in which [^{3}H]TG production by apo E–deficient and control cells was compared. On average, the cellular [^{3}H]TG content was 30% (without oleate) and 24% (with oleate) higher in apo E–deficient than in control hepatocytes, after 3 h of incubation. However, these differences were not statistically significant. In contrast, the [^{3}H]TG secretion by apo E–deficient cells was decreased significantly, by 60% in the absence (Fig. 4 *C*) and 73% in the presence of oleate (Fig. 4 *D*) compared with control cells. Similar results were obtained when [^{3}H]oleate was used to label the newly synthesized lipids.

Measurement of the incorporation of [^{14}C]acetate into cellular cholesterol, indicative for cholesterol biosynthesis, did not reveal any significant difference in this respect between apo E–deficient and control cells, either in the absence or presence of oleate in the incubation media (data not shown).

Discussion

Production of VLDL by the liver is an important determinant of plasma lipid levels in humans. It is well-established that apo B is required for the assembly and secretion of these TG-rich lipoproteins by liver cells, and that each particle contains a single apo B molecule (32). Although controversies still exist, most of the available data, as summarized in recent reviews (33–36), indicate that regulation of apo B secretion is primarily a posttranslational event governed by the efficiency of translocation of the protein across the endoplasmic reticulum membrane and its association with lipids. Excess of apo B that does not associate appropriately with lipids inside the endoplasmic reticulum lumen is degraded rapidly. There is a large body of experimental evidence indicating that both the availability of core (TG, cholesterol ester) and surface lipid (phospholipid) can become rate-limiting for VLDL production. Furthermore, several studies have shown that the microsomal triglyceride transfer protein is essential for adequate lipoprotein formation (37–39). In addition, other proteins have been identified that

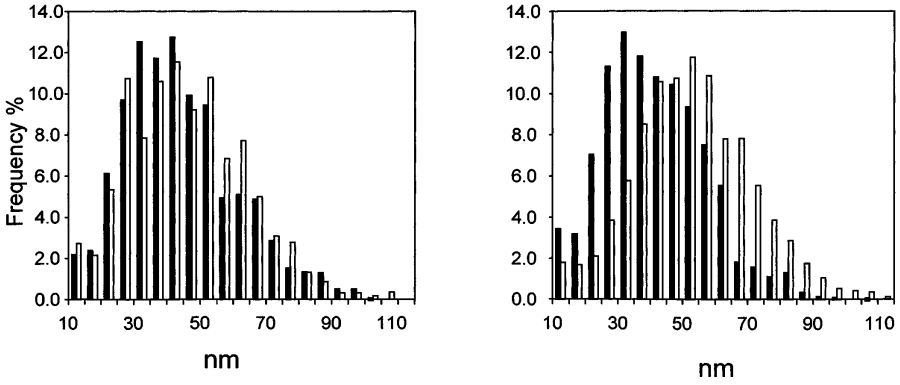

Figure 2. Frequency distribution of particle diameter of VLDL produced by apo E–deficient and control mouse hepatocytes. apo E–deficient (*black bars*) and control (C57BL/6J) mouse (*white bars*) hepatocytes were cultured for 24 h in the absence (*left panel*) or presence (*right panel*) of 0.75 mM oleate, as described in Methods. Lipoproteins ($d < 1.006$ g/ml) were isolated from the media by ultracentrifugation and studied by electron microscopy, as described in Methods. Values are expressed as the mean of four independent VLDL preparations per group. SD bars are not shown for clarity reasons. In each preparation, ~ 500 particles were analyzed.

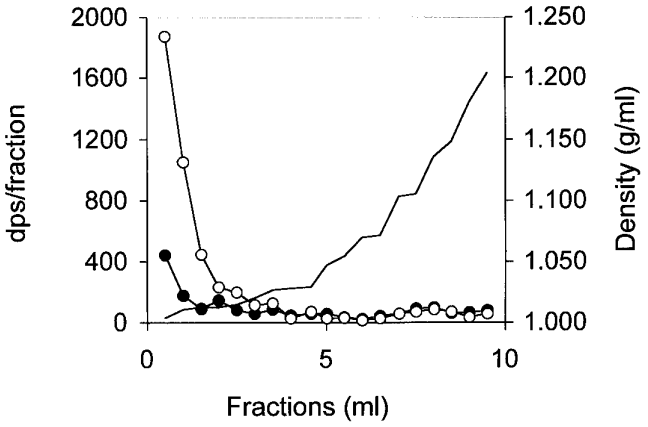

Figure 3. Density gradient ultracentrifugation profiles of radiolabeled lipids accumulated in the culture medium of primary hepatocytes isolated from control (*open symbols*) and apo E–deficient (*closed symbols*) mice. Hepatocytes were incubated in serum-free media containing [^{3}H]glycerol and 0.75 mM oleate complexed with BSA (0.25 mM) for 24 h. After incubation, the media were collected, centrifugated to remove any cell debris, and dialyzed extensively. Immediately before ultracentrifugation, 0.5 ml of normal human plasma was added as a carrier. Fractions were collected, extracted, and assayed for radioactivity as described in Methods. The data represent the mean of three independent experiments per group. *Solid line*, Average density of the collected fractions. *dps*, Desintegrations per second.

may play a role in the assembly of VLDL (for a review, see reference 35).

In this study, data are presented that identify apo E as an additional factor involved in the regulation of VLDL assembly and secretion by hepatocytes. A pronounced reduction in VLDL-TG secretion by apo E–deficient liver cells was observed in three different experimental systems, i.e., in the intact animal, in the isolated perfused liver, and in hepatocytes in primary culture. It could be argued that the decreased VLDL-TG secretion by apo E–deficient hepatocytes is not directly related to the lack of apo E, but rather to aspecific changes in the livers of apo E–deficient mice as induced by profound hypercholesterolemia. To address this possibility, in vivo hepatic TG production studies were performed with apo E–deficient mice in which the hypercholesterolemia was effectively reversed by transplantation of wild-type bone marrow. These studies showed that hepatic TG production is still severely impaired in bone marrow–transplanted apo E–deficient mice (Table I). In addition, no differences were observed between cultured apo E–deficient and control hepatocytes with respect to cell viability, protein synthesis and secretion, and LDH leakage. Altogether, these data indicate strongly that the reduced TG secretion by apo E–deficient liver cells is caused primarily by the absence of apo E.

To gain more insight into the mechanisms underlying reduced TG secretion by apo E–deficient liver cells, nascent VLDL-TG secretion studies were performed using isolated perfused mouse livers and cultured hepatocytes. One of the major advantages of the perfused liver model is that the hepatocytes maintain their secretory polarity, a feature that is obviously lost in cell culture. Secretion of VLDL-TG by livers from apo E–deficient mice was reduced by 50% compared with control mice. In addition, perfused mouse livers of either strain produced nascent VLDL particles with similar lipid composition (Table II), indicating that apo E–deficient mouse livers essentially produce normal TG-rich VLDL.

Much of our knowledge of the VLDL production process is derived from in vitro studies with hepatoma cell lines and primary hepatocyte cultures. Human (HepG2) and rat (McA-RH7777) hepatoma cell lines (15, 16) as well as SV40 large T antigen–immortalized human hepatocytes (40) typically secrete apo B–containing particles in the LDL-density range, probably as a consequence of their dedifferentiated state, in which a number of liver-specific functions are lost (40). In contrast, short-term primary cultures of rat (41), human (28), and

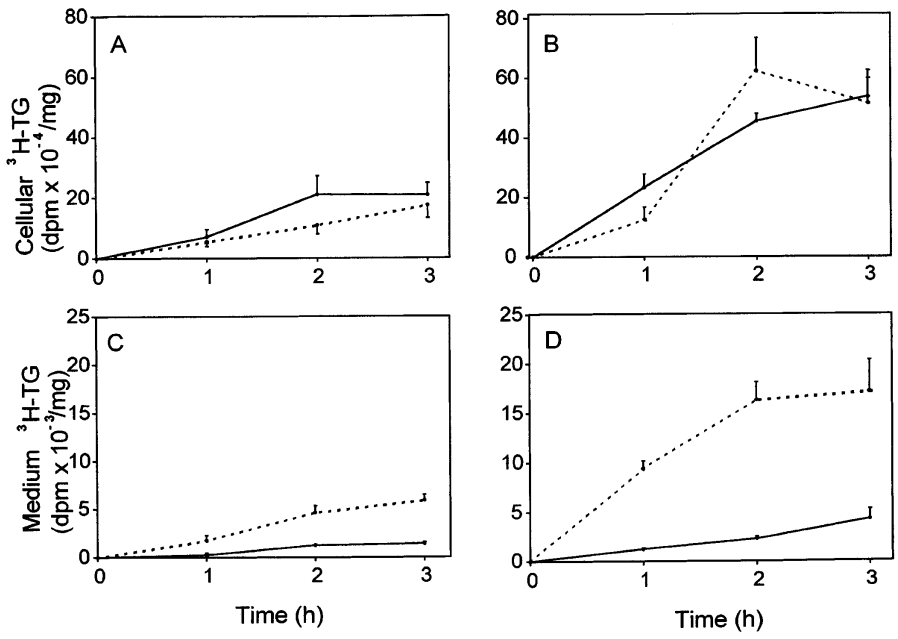

Figure 4. Time-dependent accumulation of [^{3}H]TG in apo E–deficient and control hepatocytes and secretion of [^{3}H]TG-VLDL by these cells. After their isolation, apo E–deficient (*solid line*) and control (C57BL/6J) hepatocytes (*dotted line*) were cultured overnight in FCS and hormone-containing media. Cells were washed thoroughly and incubated for an additional 4 h in FCS and hormone-free medium. Subsequently, cells were incubated with medium containing BSA in the absence (*A* and *C*) or presence (*B* and *D*) of 0.75 mM oleate. [^{3}H]Glycerol was added to label newly synthesized TG, as outlined in Methods. At the indicated time points, cells and media were harvested, and the accumulation of cellular [^{3}H]TG (*A* and *B*) and secretion of [^{3}H]TG-VLDL (*C* and *D*) were determined as described in Methods. A representative experiment is shown in which cells isolated from a single apo E–deficient mouse and a control mouse were studied simultaneously. Values are expressed as the mean±SD of triplicate determinations at each time point.

murine (20) hepatocytes secrete primarily VLDL, reflecting more closely the in vivo situation. The data depicted in Fig. 3 demonstrate that apo E–deficient hepatocytes in primary culture secrete newly synthesized lipids primarily in particles of the VLDL-density range, similar to the situation observed for wild-type control cells.

Experiments with apo E–deficient hepatocytes in culture showed an impaired VLDL-TG secretion, both when mass secretion was determined over a 24-h period (Fig. 1) and when secretion of newly synthesized TG from radiolabeled precursors was measured (Fig. 4, *C* and *D*). This impaired VLDL-TG secretion by apo E–deficient hepatocytes was not likely to be caused by decreased intracellular TG synthesis, since the TG synthesis rate as measured by [^{3}H]glycerol incorporation was similar for apo E–deficient and control cells (Fig. 4, *A* and *B*). In addition, apo E–deficient cells showed significantly higher TG and cholesterol contents compared with cells from wild-type mice (Table III). This is consistent with our previous in vivo observations (17), although the difference in hepatic TG content was larger under in vivo conditions. This may be due to selective isolation of relatively lipid-poor cells from periportal areas (zone 1), since it was found that TG-containing fat droplets are localized predominantly in perivenous (zone 3) hepatocytes in apo E–deficient livers (17). Alternatively, the lipid-laden zone 3 cells may have been lost more easily during isolation and subsequent washing procedures.

In the absence of oleate, cells from apo E–deficient mice secreted less VLDL-TG, whereas the VLDL particles were of similar size and composition as those secreted by cells from control mice (Figs. 1 and 2). These data point to a significant reduction in the number of VLDL particles secreted by apo E–deficient hepatocytes. Since each VLDL particle contains a single molecule of apo B, secretion of this apolipoprotein must also be reduced. However, in the presence of oleate, VLDL particles formed by apo E–deficient cells were significantly smaller than those produced by control cells (Fig. 2). A simple calculation shows that the 10.7-nm difference in diameter between apo E–deficient and control particle size (Fig. 2) results in a 2.2-fold larger particle volume for VLDL produced by control cells. This value relates reasonably well to the 2.6-fold higher production of core lipids by control cells under these conditions (Fig. 1). Thus, under oleate-stimulated conditions, it is suggested that apo E–deficient hepatocytes secrete smaller VLDL particles, but their overall number is not affected. Clearly, further studies are necessary to establish the modulating effect of apo E on VLDL particle size and secretion. Such experiments, including detailed analysis of apo B synthesis and secretion, are currently being performed in our laboratory.

Previous studies have shown that the synthesis rate of cholesterol in the liver may affect VLDL secretion (42). In this study, the incorporation of [^{14}C]acetate into cellular cholesterol was similar in control and apo E–deficient cells both in the absence and presence of oleate in the incubation medium. These results indicate that the impaired VLDL production by apo E–deficient hepatocytes observed in this study is not caused by differences in cholesterol biosynthesis.

How does apo E influence VLDL particle assembly and secretion? As outlined in the Introduction, Fazio et al. (15) have reported an enhanced association of apo E with large, apo B–containing lipoproteins during stimulation of lipogenesis in HepG2 cells, while the total amount of apo E secretion did not change. Similar findings were also reported in a study by Davis et al. (43) using rat hepatocytes in primary culture. Fazio and Yao (16) speculated that this increased association reflects a physical preference of apo E for the larger particles, providing an effective way to enrich these particles with apo E in order to facilitate subsequent carrier-mediated uptake of their remnants from blood. However, our data strongly suggest that apo E actually has a function in the assembly–secretion process at the intracellular level, in particular when lipogenesis is stimulated by the presence of oleate in the culture media. Although the exact mechanism(s) remains to be elucidated, our data seem to indicate that apo E is involved in the incorporation of (newly synthesized) TG into VLDL particles produced by mouse liver cells. At this point, it is tempting to speculate that apo E interferes with the recruitment of newly synthesized TG into the small microsomal secretion-coupled pool of TG that is suggested to be involved in the regulation of apo B secretion (44). Interestingly, Gretch et al. (45) have found recently that the expression of human apo E in insect larvae leads to progressive conversion of the endogenous lipoproteins into more buoyant species. Although there clearly are major differences in lipoprotein assembly between the insect and mammalian systems, this finding demonstrates that apo E can influence the amount of lipid assembled into lipoproteins and thereby affect their size and buoyant density. Thus, in conclusion, we propose that in addition to its well-established function in lipoprotein uptake by the liver, apo E also plays an important physiological role in lipoprotein formation by hepatocytes.

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