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Physiological relevance of apolipoprotein E recycling: studies in primary mouse hepatocytes☆

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Abstract

Studies in our laboratory have shown that a fraction of apolipoprotein (apo) E internalized by hepatocytes escapes degradation and is resecreted. Although the intracellular routing is not fully understood, our studies suggest that a portion of apoE recycles through the Golgi apparatus. Given the role of the Golgi apparatus in lipoprotein secretion and the fact that apoE modulates the hepatic secretion of very low-density lipoprotein, we hypothesized that recycling apoE has an effect on hepatic very low-density lipoprotein assembly and/or secretion. To test this hypothesis, apoE$^{-/-}$ mice were transplanted with bone marrow from wild-type mice. In this model, extrahepatic (macrophage-derived) apoE is internalized by the hepatocytes in vivo and is resecreted when the hepatocytes are placed in culture. Unexpectedly, our studies demonstrate that recycling apoE has little effect on hepatic lipid content or hepatocyte triglyceride secretion. In addition, recycling apoE has little effect on the expression of enzymes and proteins involved in lipid synthesis as well as plasma lipoprotein apoproteins. We conclude that the physiological relevance of apoE recycling may not be related to cell-specific functions, such as lipoprotein assembly in the liver. Rather, recycling may provide a mechanism for modulating general cellular effects such as intracellular cholesterol transport or cholesterol efflux.

1. Introduction

Apolipoprotein (apo) E is a 34-kd protein that plays a key role in the metabolism of lipids and lipoproteins. Extracellularly, apoE mediates the internalization of lipoprotein particles by serving as a ligand for the low-density lipoprotein (LDL) receptor [1,2], the LDL receptor–related protein [3,4], and heparan sulfate proteoglycans, the latter either directly [5,6] or via an LDL receptor–related protein–dependent mechanism [7,8]. Intracellularly, apoE modulates lipid metabolism [9,10] and is believed to play a role in the routing of internalized lipoprotein remnants [11,12]. In addition, apoE is involved in both the assembly [13,14] and secretion [15,16] of very low-density lipoproteins (VLDLs) in hepatocytes. In macrophages, apoE also plays a critical role in cholesterol efflux [17-20]. Based on the multiple and critical roles for apoE in the metabolism of lipids and lipoproteins, we and others hypothesized and showed that a fraction of apoE internalized with lipoproteins escapes lysosomal degradation and is resecreted. We and others also hypothesized that the sparing of apoE would have an impact on specific cellular functions, such as lipoprotein assembly in hepatocytes, cholesterol efflux in macrophages, or cholesterol routing in any cell type. The evidence for this has not yet been provided.

We reported that apoAI stimulates apoE resecretion from hepatocytes, thus, suggesting a role for recycling apoE in high-density lipoprotein (HDL) metabolism [21]. We also demonstrated the presence of radiolabeled apoE in Golgi apparatus–rich fractions isolated from mice injected with iodinated VLDL [22] and we found apoE in hepatic Golgi fractions of apoE$^{-/-}$ mice that had been transplanted with

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marrow from wild-type mice [23]. These data suggested that a portion of apoE recycles through the Golgi apparatus. Given the fact that apoE modulates the hepatic secretion of VLDL and the role of the Golgi apparatus in lipoprotein secretion, our observations suggested that recycling apoE might affect hepatic assembly and/or secretion.

In the present study, we used apoE$^{-/-}$ mice transplanted with bone marrow from wild-type mice as an in vivo model of recycling apoE in hepatocytes. In this model, extrahepatic (macrophage derived) apoE is internalized by the hepatocytes and is resecreted when the hepatocytes are placed in culture. Thus, the bone marrow–transplanted apoE$^{-/-}$ mouse provides a model to assess the effects of recycling apoE on hepatocyte functions and properties. Unexpectedly, our studies demonstrate that recycling apoE has little effect on hepatic lipid content or hepatic triglyceride secretion whether measured in vivo or in hepatocytes in culture. In addition, recycling apoE has little effect on the expression of enzymes involved in lipid synthesis as well as plasma lipoprotein apoproteins. We conclude that the physiological relevance of apoE recycling may not be related to cell-specific functions, such as lipoprotein assembly in the hepatocyte. Rather, recycling may provide a mechanism for modulating general cellular effects such as intracellular cholesterol transport or cholesterol efflux.

2. Materials and methods

2.1. Animals

ApoE-deficient (apoE$^{-/-}$) mice on the C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, Me). A colony of C57BL/6J mice was established in our animal facility. All mice were kept on a 12-hour light-dark cycle and were fed a normal mouse chow diet (RP5015; PMI Feeds Inc, St Louis, Mo). Food and water were available ad libitum. All animal procedures were carried out in accordance with institutional guidelines with approval from the Animal Care Committee of Vanderbilt University.

2.2. Bone marrow transplantation

Bone marrow transplantation (BMT) was carried out as described previously [24]. One week before and 2 weeks after BMT, neomycin (100 mg/L) and polymyxin B sulfate (10 mg/L; Sigma, St Louis, Mo) were added to the water. Bone marrow was collected from donor mice by flushing femurs with RPMI 1640 media containing 2% fetal bovine serum and 10 U/mL heparin (Sigma). Recipient mice were lethally irradiated (9 Gy), and 4 hours later, 5×10^6 bone marrow cells in 0.3 mL were transplanted by tail vein injection. Animals were used for studies from 6 to 12 weeks after transplantation.

2.3. Hepatic lipid analyses

Pieces of liver (~100 mg), taken from the same lobe of each mouse, were weighed and homogenized in chloroform/methanol (2:1 vol/vol), and lipids were recovered by the method of Folch et al [25]. Aliquots of the lipid extract were separated into individual lipid classes by thin-layer chromatography using Silica Gel 60 A plates developed in petroleum ether/ethyl ether/acetic acid (80:20:1). Lipid spots were visualized using rhodamine 6G. Phospholipids and triglycerides were scraped from the plates, and the fatty acids were hydrolyzed and methylated using BF_3/methanol as described by Morrison and Smith [26]. Methylated fatty acids were analyzed on an HP 5890 gas chromatograph using a capillary column (Supelcowax 10, 0.25 mm × 30 m, 0.25 μm film, Supelco, Bellefonte, Pa). Fatty acid methyl esters were identified by comparing the retention times to those of known standards. Odd chain fatty acids were added as standards to permit the quantitation of the amount of lipid in the sample.

To determine unesterified cholesterol, 5-α-cholestane was added to an aliquot of the total lipid extract. The sample was dried under nitrogen, brought up in 50 μL of hexane, and quantitated by gas chromatography using a DB-17 column (15 m × 0.53 mm, 1 μm film, Agilent, Wilmington, Del). To determine total cholesterol, a second aliquot, containing 5-α-cholestane, was saponified using 1N KOH in 90% methanol. The nonsaponifiable sterol was extracted with hexane and analyzed by gas chromatography. The difference in mass between total cholesterol and unesterified cholesterol was assumed to be esterified cholesterol. The latter number was multiplied by 1.67 to obtain mass of cholesterol esters.

2.4. Isolation and culture of primary mouse hepatocytes

Hepatocytes were isolated as described previously [23]. Cells were plated onto 6-well (35 mm) BioCoat collagen IV–coated dishes (Becton-Dickinson, Franklin Lakes, NJ) at a density of 4.5×10^5 viable cells per milliliter in low-glucose DMEM (Invitrogen Corporation, Carlsbad, Calif) containing 1% bovine serum albumin (BSA), 0.8 mmol/L oleate, 0.167 μg/mL insulin (4 mU/mL), 0.02 μg/mL dexamethasone, 100 U penicillin, and 100 μg streptomycin/mL. Four hours after plating, the media along with nonadherent cells was removed, the cells were washed, and fresh media was added. Cells were maintained at 37°C in 5% CO_2.

2.5. Glycerolipid synthesis and secretion

Hepatocytes were cultured for 16 hours in low-glucose DMEM containing 1% BSA and 0.8 mmol/L oleate. Fresh media, containing [^{3}H] glycerol (4.4 μCi, 25 μmol/L final concentration; Amersham Biosciences, Piscataway, NJ), was added to each well, and the cells were incubated for 1, 2, or 3 hours. All incubations were performed in duplicate and terminated by placing the culture plates on ice. Media was collected and centrifuged at 4000 rpm for 2 minutes to remove any suspended cells. Cells were washed 3 times with ice-cold phosphate-buffered saline and harvested in phosphate-buffered saline.

Table 1
Serum lipid concentrations

	C57BL/6	ApoE$^{-/-}$	ApoE$^{+/+}$→ ApoE$^{-/-}$	ApoE$^{-/-}$→ ApoE$^{-/-}$
Triglycerides	107 ± 11[a]	171 ± 21[a,b]	93 ± 10[b]	137 ± 25
Cholesterol	67 ± 4[c]	280 ± 20[c,d]	78 ± 6[d]	319 ± 17[c]

Serum lipids were analyzed by enzymatic assays on 6 to 9 mice per group and are expressed as mean ± SD in mg/dL. Values sharing the same superscript letter are significantly different at [a]$P < .05$, [b]$P < .01$, and [c,d]$P < .001$.

2.6. Cellular and VLDL lipid content

Radiolabeled lipids from media and cells were extracted using the Folch procedure [25]. Before extraction, an aliquot of the suspended cells was taken for protein determination. The lipid classes were separated by thin-layer chromatography as described previously. The lipid bands were identified by iodine staining, and the appropriate bands were scraped from the plates into scintillation vials. Radioactivity was determined using Biosafe II scintillation fluid (Research Products International Corp, Mt Prospect, Ill) and a Beckman LS6800 scintillation counter (Beckman, Palo Alto, Calif).

2.7. RNA isolation and Northern blot analyses

Total RNA was extracted from liver with Trizol reagent (Invitrogen) as per the manufacturer's instructions. RNA (20 μg) from 3 individual mouse livers in each of the 3 groups was electrophoresed in 1% agarose gel containing formaldehyde and transferred to a Hybond N membrane (Amersham Biosciences). The cDNA probes were a generous gift from Hitoshi Shimano (University of Tokyo, Tokyo, Japan) and were labeled with [α-^{32}P]dCTP using the Prime-It II DNA labeling system kit (Amersham Biosciences). The membranes were hybridized with the radiolabeled probe in hybridization buffer (42% formamide, 8% Denhardt's 50×, 40 mmol/L Tris-HCl, 350 mmol/L NaCl, 1% sodium dodecyl sulfate) at 42°C. Blots were exposed to film, and scanned using a BioRad GS700 Imaging Densitometer, and relative messenger RNA (mRNA) levels estimated using Molecular Analyst software. Quantification of mRNA of interest was made by normalizing to the signal generated from G3PDH mRNA.

2.8. Biochemical analyses

Serum triglycerides and cholesterol were measured using enzymatic assays adapted to microtiter plates (Raichem, San Diego, Calif). Cell protein was determined by the bicinchoninic acid method (Pierce, Rockford, Ill), modified to eliminate interference by lipid [27] using BSA as standard.

2.9. Statistics

Data were analyzed by analysis of variance using GraphPad InStat (GraphPad Software Inc, San Diego, Calif), and comparisons were made using the Tukey-Kramer multiple comparisons test.

3. Results

3.1. Serum lipids

The serum lipids in the 4 groups of animals are shown in Table 1. ApoE$^{-/-}$ mice had elevated cholesterol and triglyceride levels compared with wild-type mice. Transplantation with marrow from wild-type mice (apoE$^{+/+}$ → apoE$^{-/-}$) normalized cholesterol and triglyceride levels as described previously [24]. Serum triglycerides in apoE$^{-/-}$ mice transplanted with marrow from apoE$^{-/-}$ mice (apoE$^{-/-}$ → apoE$^{-/-}$) were unchanged compared with apoE$^{-/-}$ mice; however, serum cholesterol levels in this group were elevated compared with apoE$^{-/-}$ mice.

3.2. Hepatic lipids and fatty acids

The livers from apoE$^{-/-}$ mice had slightly elevated levels of triglyceride compared with C57Bl/6 mice, although the increase did not reach statistical significance (Fig. 1A). ApoE$^{+/+}$ → apoE$^{-/-}$ and apoE$^{-/-}$ → apoE$^{-/-}$ mice had significantly elevated levels of hepatic triglyceride compared with both C57BL/6 and apoE$^{-/-}$ controls. However, there were no differences in hepatic phospholipid levels among the 4 groups of mice. Total cholesterol was significantly elevated in livers from apoE$^{-/-}$ mice and both groups of mice undergoing BMT compared with C57BL/6 mice (Fig. 1B). Unesterified cholesterol was significantly elevated in livers from apoE$^{-/-}$ mice compared with livers from C57BL/6 mice, and cholesteryl ester content was significantly elevated in apoE$^{+/+}$ → apoE$^{-/-}$ and apoE$^{-/-}$ → apoE$^{-/-}$ mice compared with C57BL/6 and apoE$^{-/-}$ mice (Fig. 1B).

The fatty acid composition of hepatic triglycerides and phospholipids in the 4 groups of mice are shown in

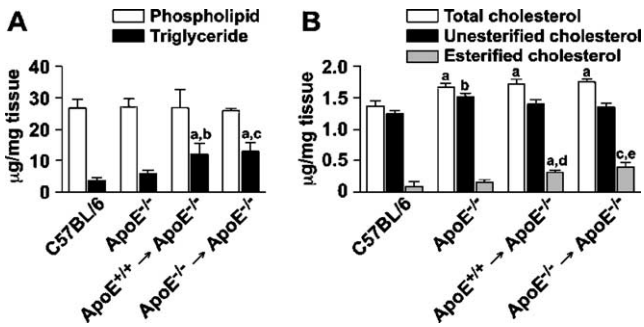

Fig. 1. Hepatic lipid content of C57BL/6, apoE$^{-/-}$, and bone marrow–transplanted mice. Pieces of liver were collected and lipids extracted and analyzed as described in Materials and Methods. The data are expressed as mean ± SD (n = 9) for C57BL/6, apoE$^{-/-}$, and apoE$^{+/+}$ → apoE$^{-/-}$; n = 6 for apoE$^{-/-}$ → apoE$^{-/-}$. A, Glycerolipids. [a]$P < .05$, significantly different from C57BL/6. [b]$P < .05$ and [c]$P < .01$, significantly different from apoE$^{-/-}$. B, Cholesterol. [a]$P < .01$, [b]$P < .05$, and [c]$P < .001$, significantly different from C57BL/6. [d]$P < .05$ and [e]$P < .01$, significantly different from apoE$^{-/-}$.

Table 2
Fatty acid composition of hepatic triglycerides

Fatty acid	C57BL/6	ApoE$^{-/-}$	ApoE$^{+/+}$ → ApoE$^{-/-}$	ApoE$^{-/-}$ → ApoE$^{-/-}$
16:0	28.6 ± 2.5	27.4 ± 2.0	24.2 ± 2.8	24.1 ± 2.1
16:1	3.3 ± 1.9	3.5 ± 0.8	2.6 ± 1.2	3.0 ± 0.8
18:0	2.6 ± 2.0	3.3 ± 1.3	2.9 ± 1.4	2.7 ± 0.7
18:1	33.2 ± 1.2	30.2 ± 1.5	32.1 ± 2.3	31.8 ± 3.9
18:2	23.9 ± 3.4	25.0 ± 2.6	27.1 ± 2.3	26.5 ± 2.8
20:3	n.d.	0.3 ± 0.4	0.3 ± 0.4	0.3 ± 0.4
20:4	1.0 ± 1.5	1.8 ± 1.3	1.7 ± 1.1	2.1 ± 0.6
20:5	0.2 ± 0.5	0.4 ± 0.6	0.5 ± 0.8	0.7 ± 0.8
22:5	0.2 ± 0.7	1.8 ± 0.2	1.4 ± 1.1	1.7 ± 0.9
22:6	7.0 ± 1.2	7.4 ± 1.3	7.0 ± 1.8	6.6 ± 1.7

Data are expressed as percentage of total fatty and represent the mean ± SD from 6 to 9 different assays. n.d. indicates not detected.

Tables 2 and 3. The major fatty acids in hepatic triglycerides were 16:0, 18:1 and 18:2, whereas the major fatty acids in the hepatic phospholipids were 16:0, 18:0, 18:2, 20:4, and 22:6. There were no differences in any of the fatty acids across the 4 groups of mice.

3.3. Glycerolipid synthesis and secretion by primary hepatocytes in culture

The synthesis and secretion of glycerolipids by primary hepatocytes from C57BL/6, apoE$^{-/-}$, and apoE$^{+/+}$ → apoE$^{-/-}$ mice were studied after incubation with [^{3}H]-glycerol (Fig. 2). Hepatocytes from C57BL/6 and apoE$^{-/-}$ mice demonstrated similar time-dependent accumulation of [^{3}H]-triglyceride (Fig. 2A). The synthesis of [^{3}H]-triglyceride in apoE$^{+/+}$ → apoE$^{-/-}$ mice tended to be lower than in hepatocytes from C57BL/6 and apoE$^{-/-}$ mice; however, accumulation of newly synthesized triglyceride in hepatocytes from transplanted mice was significantly decreased ($P < .05$) only at the 3-hour time point (Fig. 2A). The synthesis of glycerol-containing phospholipids followed a similar pattern as triglyceride synthesis with C57BL/6 and apoE$^{-/-}$ mice displaying similar synthetic rates (Fig. 2C). As with the synthesis of [^{3}H]-triglyceride, the synthesis of [^{3}H]-phospholipid in hepatocytes from apoE$^{+/+}$ → apoE$^{-/-}$ mice tended to be less than in hepatocytes from C57BL/6 and apoE$^{-/-}$ mice, but accumulation of [^{3}H]-phospholipid in hepatocytes from transplanted mice was significantly decreased only at the 3-hour time point (Fig. 2C).

Table 3
Fatty acid composition of hepatic phospholipids

Fatty acid	C57BL/6	ApoE$^{-/-}$	ApoE$^{+/+}$ → ApoE$^{-/-}$	ApoE$^{-/-}$ → ApoE$^{-/-}$
16:0	23.0 ± 1.1	22.8 ± 1.1	22.3 ± 0.9	22.1 ± 0.9
16:1	1.4 ± 0.1	1.2 ± 0.4	1.0 ± 0.2	1.0 ± 0.3
18:0	13.7 ± 1.6	14.7 ± 1.9	15.9 ± 2.1	14.8 ± 1.1
18:1	7.7 ± 0.3	6.7 ± 0.7	6.9 ± 0.7	7.0 ± 0.9
18:2	17.1 ± 1.1	17.9 ± 1.5	17.0 ± 1.2	18.1 ± 0.7
20:3	2.4 ± 0.4	1.8 ± 0.5	2.0 ± 0.4	1.7 ± 0.2
20:4	13.9 ± 1.2	13.8 ± 1.1	15.0 ± 1.6	14.6 ± 0.5
20:5	2.3 ± 0.5	2.6 ± 0.3	1.6 ± 0.4	1.9 ± 0.4
22:5	1.1 ± 0.2	1.2 ± 0.3	1.0 ± 0.1	1.1 ± 0.1
22:6	17.2 ± 2.1	17.3 ± 1.5	17.2 ± 1.6	17.3 ± 0.3

Data are expressed as percentage of total fatty and represent the mean ± SD from 6 to 9 different assays.

The rate of secretion of triglyceride from hepatocytes from apoE$^{-/-}$ mice was decreased significantly ($P < .05$) when compared with hepatocytes from C57BL/6 mice (Fig. 2B). The introduction of apoE via BMT and its subsequent recycling in hepatocytes did not reverse the decreased secretion of triglyceride noted in the apoE-deficient cells. In fact, triglyceride secretion in hepatocytes from apoE$^{+/+}$ → apoE$^{-/-}$ mice was significantly decreased when compared with hepatocytes from either C57BL/6 or apoE$^{-/-}$ mice ($P < .001$ and $P < .05$, respectively). The secretion of newly synthesized phospholipids was much less than the secretion of newly synthesized triglyceride (Fig. 2D). However, there were no significant differences among hepatocytes from the 3 groups of mice.

3.4. Expression of mRNA

The effect of recycling apoE on the hepatic expression of a number of enzymes and regulatory proteins involved in cellular lipid homeostasis was determined by Northern blot analyses (Fig. 3). We found no difference in expression of fatty acid synthase (FAS), stearyl-CoA desaturase–1

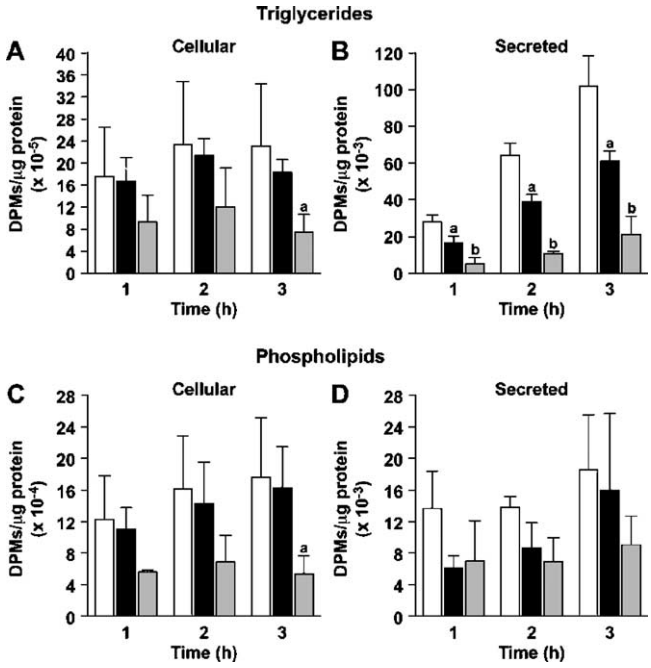

Fig. 2. Accumulation and secretion of [^{3}H]-triglyceride and [^{3}H]-phospholipid by hepatocytes from C57BL/6, apoE$^{-/-}$, and apoE$^{+/+}$ → apoE$^{-/-}$ mice. Primary hepatocytes were isolated as described in Materials and Methods. Cells were incubated with [2-^{3}H]-glycerol for 1 to 3 hours, and radioactivity in triglyceride and phospholipids in the cells (A, C) and media (B, D) was determined. The results represent the mean ± SD. White bars indicate C57BL/6 (n = 3); black bars, apoE$^{-/-}$ (n = 3); gray bars, apoE$^{+/+}$ → apoE$^{-/-}$ (n = 4). [a]$P < .05$, significantly different from C57BL/6 and apoE$^{-/-}$ (A). [a]$P < .05$, significantly different from C57BL/6; [b]$P < .001$ and [b]$P < .05$, significantly different from C57BL/6 and apoE$^{-/-}$ (B). [a]$P < .05$, significantly different from C57BL/6 and apoE$^{-/-}$ (C).

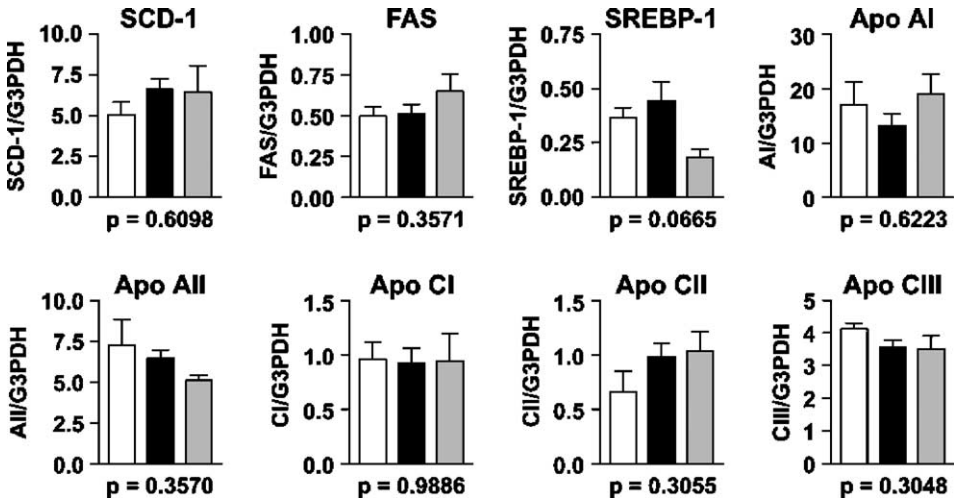

Fig. 3. Expression of hepatic enzymes and apoproteins in C57BL/6, apoE$^{-/-}$, and apoE$^{+/+}$ → apoE$^{-/-}$ mice. Total RNA was extracted from the liver of 3 mice in each of the 3 groups, electrophoresed in 1% agarose gels containing formaldehyde, and transferred to a Hybond N membrane as described in Materials and Methods. cDNA probes were labeled with [α-^{32}P]dCTP and hybridized to the membranes at 42°C. Blots were exposed to film, and the films were scanned using a BioRad GS700 Imaging Densitometer. Relative mRNA levels were estimated using Molecular Analyst software. Quantification of the mRNA of interest was made by normalizing to the signal generated from G3PDH mRNA. The results are expressed as the mean ± SD, and *P* values for each group are noted. White bars indicate C57BL/6; black bars, apoE$^{-/-}$; gray bars, apoE$^{+/+}$ → apoE$^{-/-}$.

(SCD-1), or sterol regulatory element binding protein 1 (SREBP-1) among the 3 groups of animals. Expression of SREBP-1 in the BMT animals was lower than in C57BL/6 and apoE$^{-/-}$ mice but this difference did not reach significance (P = .0665). There were no differences in the expression of apoAI, apoAII, apoCI, apoCII, or apoCIII among the 3 groups of mice.

4. Discussion

Previous studies from our laboratory have shown that primary cultures of hepatocytes from apoE$^{-/-}$ mice, transplanted with bone marrow from wild-type mice, secrete apoE for up to 45 hours after hepatocyte isolation [23], providing definitive evidence for the recycling of apoE within the hepatocyte. Furthermore, our studies demonstrated that hepatocytes isolated from BMT mice retained nearly 70% of the apoE internalized in vivo after 20 hours in culture. In the present study we used this BMT model to assess the physiological relevance of apoE recycling in hepatocytes. Our studies demonstrate that apoE recycling in hepatocytes from BMT mice had no effect on hepatic lipids or on the accumulation or secretion of triglyceride by primary hepatocytes in culture. In addition, apoE recycling had no effect on the expression of enzymes and proteins involved in hepatic lipid synthesis or on apoproteins produced by the liver. We conclude that the recycling of apoE in hepatocytes from BMT mice has little effect on hepatic lipid and lipoprotein metabolism and that the physiological relevance of apoE recycling may not be related to mass action and cell-specific functions, such as lipoprotein assembly in the liver, but rather to broader and finer homeostatic functions, such as cellular cholesterol routing and efflux.

Bone marrow transplantation has proven to be an excellent tool for studying both the metabolic and pathological effects of apoE [24,28-32]. Our group was the first to demonstrate that introduction of apoE into apoE-deficient mice via BMT leads to normalization of serum cholesterol levels by providing a ligand for the receptor-mediated removal of remnant lipoproteins [24]. This normalization occurs although plasma levels of apoE are one tenth the levels found in wild-type mice. As a result of the lower plasma lipid levels and alterations in macrophage lipid metabolism, apoE$^{+/+}$ → apoE$^{-/-}$ mice showed virtually complete protection from diet-induced atherosclerosis. In subsequent studies we demonstrated the presence of apoE in hepatic Golgi-rich fractions from apoE$^{+/+}$ → apoE$^{-/-}$ mice [23], and we reported that hepatocytes isolated from these transplanted mice secrete apoE when placed in primary culture. Therefore, the BMT model provides an excellent tool to investigate the physiological effects of apoE recycling in the hepatocyte.

Kuipers et al [10] reported that hepatic triglyceride content was elevated in apoE$^{-/-}$ mice. In our studies, hepatic triglyceride content tended to be higher in apoE$^{-/-}$ mice, but it was not significantly elevated (Fig. 1A). Bone marrow transplantation with wild-type marrow resulted in a significant increase in hepatic triglycerides. In fact, hepatic triglycerides and cholesteryl esters were significantly elevated in livers from both groups of bone marrow–transplanted mice (Fig. 1). These observations suggest that the BMT procedure leads to accumulation of neutral lipid within the liver. Most likely this increase in neutral lipid is related to the irradiation of the BMT mice. Whole body irradiation has been shown to lead to accumulation of triglycerides in livers of rats [33]. Bone marrow transplantation had no effect on total hepatic cholesterol levels compared with apoE$^{-/-}$ mice, but there

was a tendency for unesterified cholesterol levels to be reduced in the livers from the transplanted animals (Fig. 1B).

Total triglyceride accumulation was similar in hepatocytes from C57BL/6, apoE$^{-/-}$, and BMT mice (Fig. 2A) except at the 3-hour time point at which triglyceride accumulation in hepatocytes from BMT mice was significantly decreased compared with the other 2 groups. Triglyceride secretion from apoE-deficient hepatocytes was approximately 60% of that found with hepatocytes from C57BL/6 mice (Fig. 2B). A number of studies have shown that hepatic triglyceride secretion is decreased in apoE-deficient mice [15,16,34]. Furthermore, reintroduction of apoE by cross-breeding with wild-type animals or by adenoviral techniques leads to normalization of hepatic triglyceride secretion rates [16], and overexpression of apoE stimulates hepatic VLDL secretion [34-36]. In our studies the reintroduction of apoE via BMT did not increase triglyceride secretion. This might suggest that apoE is routed to a site in the secretory pathway distal to VLDL assembly or triglyceride production. Alternatively, introduction of apoE via BMT, an intervention that reconstitutes only 10% of the total serum apoE, may not provide intracellular concentrations of apoE needed to affect triglyceride production and VLDL secretion from hepatocytes. It is also possible that bone marrow transplantation decreases hepatic triglyceride secretion to the point of masking a possible minor effect of extrahepatic apoE. If this were the case, one would predict that triglyceride secretion from hepatocytes of apoE$^{-/-}$ mice transplanted with apoE$^{-/-}$ marrow would be lower than that observed in hepatocytes from mice transplanted with wild-type marrow. However, previous studies in our laboratory have shown that the in vivo triglyceride secretion rates in apoE$^{-/-}$ mice transplanted with wild-type or apoE$^{-/-}$ marrow are identical [23]. Therefore, we conclude that apoE introduced by BMT does not affect hepatic triglyceride secretion.

We explored the possibility that recycling apoE could alter the hepatic expression of proteins required in lipid synthesis. We studied FAS, SCD-1, and SREBP-1, as well as several major apoproteins. Fatty acid synthase is a key enzyme in the synthesis of fatty acids; SCD-1 is important in the production of monounsaturated fatty acids; and SREBP-1 plays a key role in hepatic transcriptional regulation of lipogenic enzymes, including FAS and SCD-1. Deletion of SREBP-1 gene severely impairs the induction of hepatic mRNAs of fatty acid synthetic genes, such as acetyl-CoA carboxylase, FAS, and SCD-1 that was observed upon refeeding in wild-type mice [37]. In our study, there were no significant differences in the expression of enzymes or apoproteins in the livers between the different groups (Fig. 3). These results provide evidence against a role for recycling apoE in triglyceride assembly and lipoprotein secretion in the liver. The lack of an effect may simply reflect suboptimal concentrations of intracellular recycling apoE. Although the plasma concentration of apoE is sufficient to normalize plasma lipid levels, the actual amount of apoE that is internalized and recycles may not be sufficient to trigger intracellular responses. In addition, the remnant lipoprotein carrying apoE to the liver in our transplant model is different from the wild-type in that it has a high content of apoAI, and apoE is fully exposed on the surface rather than being embedded in the lipoprotein. These differences could affect the extent, characteristics, and physiological effect of recycling apoE. Alternatively, apoE recycling may be involved in finer aspects of cellular cholesterol trafficking.

ApoE has been shown to recycle in a number of different cells including primary cultures of mouse hepatocytes [21,23,38], hepatoma cell lines [39,40], fibroblasts [39], macrophages [21], and CHO cells (unpublished observation). Thus, recycling does not represent a cell-specific property but seems to represent a general property of apoE. If that is the case, it would be reasonable to expect a biologic effect from recycling apoE that can be applicable to all cells, including non–lipoprotein-producing cells.

Studies in our laboratory and by Heeren et al [41] have suggested that apoE recycling is connected with HDL metabolism and cellular cholesterol efflux [21]. This suggestion is based primarily on the observations that extracellular lipid acceptors such as HDL and apoAI stimulate apoE recycling. Given the fact that apoAI and HDL have been reported to mediate the efflux of cellular lipids, this suggests that there might be a connection between the recycling of apoE and cholesterol efflux. Heeren et al [41] have shown increased cholesterol efflux in human hepatoma cells during recycling of apoE. However, cholesterol efflux studies in hepatocytes are marred by technical difficulties because of the massive background of lipoprotein cholesterol secretion and are of dubious physiological relevance, as cholesterol efflux is not crucial in cells that have both the lipoprotein and biliary routes available to get rid of mass amounts of excess cholesterol.

In summary, we conclude that the physiological relevance of apoE recycling may not be related to cell-specific functions such as lipoprotein assembly in the hepatocyte, but may provide a mechanism for modulating general cellular effects such as cholesterol efflux. Future studies will focus on the effects of apoE recycling on cholesterol efflux and on the overall impact of recycling apoE on cellular cholesterol metabolism.

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