Nascent very-low-density lipoprotein triacylglycerol hydrolysis by lipoprotein lipase is inhibited by apolipoprotein E in a dose-dependent manner

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In the present study it was investigated whether apolipoprotein (apoE) can inhibit the lipoprotein lipase (LPL)-mediated hydrolysis of very-low-density-lipoprotein (VLDL) triacylglycerols (TAGs). Previous studies have suggested such an inhibitory role for apoE by using as a substrate for LPL either plasma VLDL or artificial TAG emulsions. To mimic the *in vivo* situation more fully, we decided to investigate the effect of apoE on the LPL-mediated TAG hydrolysis by using VLDL from apoE-deficient mice that had been enriched with increasing amounts of apoE. Furthermore, since plasma VLDL isolated from apoE-deficient mice was relatively poor in TAGs and strongly enriched in cholesterol as compared with VLDL from wild-type mice, we used nascent VLDL obtained by liver perfusions. Nascent VLDL ($d < 1.006$) isolated from the perfusate of the apoE-deficient mouse liver was rich in TAGs. Addition of increasing amounts of apoE to apoE-deficient nascent VLDL effectively decreased

TAG lipolysis as compared with that of apoE-deficient nascent VLDL without the addition of apoE (63.1 ± 6.3 and $20.8 \pm 1.8\%$ of the control value at $2.7 \mu\text{g}$ and $29.6 \mu\text{g}$ of apoE/mg of TAG added respectively). Since, *in vivo*, LPL is attached to heparan sulphate proteoglycans (HSPG) at the endothelial matrix, we also performed lipolysis assays with LPL bound to HSPG in order to preserve the interaction of the lipoprotein particle with the HSPG–LPL complex. In this lipolysis system a concentration-dependent decrease in the TAG lipolysis was also observed with increasing amounts of apoE on nascent VLDL, although to a lesser extent than with LPL in solution ($72.3 \pm 3.6\%$ and $56.6 \pm 1.7\%$ of control value at $2.7 \mu\text{g}$ and $29.6 \mu\text{g}$ of apoE/mg TAGs added respectively). In conclusion, the enrichment of the VLDL particle with apoE decreases its suitability as a substrate for LPL in a dose-dependent manner.

INTRODUCTION

Apolipoprotein E (apoE), a 34 kDa protein, plays a central role in the metabolism of very-low-density-lipoprotein (VLDL) triacylglycerol (TAGs). Its most well-defined function is to mediate the high-affinity binding of chylomicron and VLDL remnants to the low-density-lipoprotein (LDL) receptor [1], the LDL-receptor-related protein (LRP) [2] and VLDL receptor [3]. In addition, apoE is postulated to play a role in the secretion–recapture process [4,5]. In this process, the binding and internalization of circulating lipoproteins by hepatic receptors is facilitated through binding of the particle to heparan sulphate proteoglycans (HSPG) and subsequent enrichment with apoE in the hepatic sinusoidal space of Disse. Furthermore, studies by Schwiegelshohn et al. [6] have shown that apoE may modulate the intracellular metabolism of TAGs and cholesterol ester in macrophages. Altogether, these studies show that apoE is a multifunctional protein involved in several steps of lipoprotein metabolism.

It has also been proposed that apoE may be involved in the lipoprotein lipase (LPL)-mediated hydrolysis of VLDL-TAGs. An inhibitory role for apoE on TAG hydrolysis was suggested by using as a substrate for LPL either plasma VLDL [7] or artificial TAG emulsions containing apoE [8,9]. However, both substrates are not fully representative of the *in vivo* situation: (i) VLDL isolated from plasma is relatively poor in TAGs owing to its exposure to LPL during its presence in the circulation and (ii)

artificial TAG emulsions are shown to be removed from the plasma with little or no preceding lipolysis [10] or have been reported to be extensively processed by LPL [9]. These differences in the LPL-mediated processing of artificial TAG emulsions may depend greatly on their individual lipid composition, since the type of non-esterified ('free') fatty acid (NEFA) and phospholipids, as well as the amounts of cholesterol in lipid emulsions have been reported to have pronounced effects on their metabolism in the circulation [11–14].

To circumvent these limitations in the present study we investigated more directly whether apoE could modulate the lipolysis of TAG-rich VLDL. Therefore, as a substrate for LPL, we used nascent apoE-deficient VLDL, obtained by perfusion of livers from apoE-deficient mice that had been enriched with increasing amounts of apoE. By this approach we clearly show that the presence of apoE on the VLDL particle inhibits TAG hydrolysis in a concentration-dependent manner.

METHODS

Animals and diets

Homozygous apoE-deficient mice were generated as previously described [15]. C57BL/6 mice were used as controls. Experiments were performed with female mice of age 8–10 weeks. All mice in the present study were housed under standard conditions with free access to water and food. Mice were fed either a regular

Abbreviations used: Apo, apolipoprotein; VLDL, very-low-density lipoprotein(s); TAG(s), triacylglycerol(s); LDL, low-density lipoprotein(s); LFC, low-fat low-cholesterol diet; LPL, lipoprotein lipase; NEFA, non-esterified ('free') fatty acid(s); HSPG, heparan sulphate proteoglycans.

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chow diet or a semisynthetic low-fat low-cholesterol (LFC) diet composed essentially as described by Nishina et al. [16] (purchased from Hope Farms, Woerden, The Netherlands). The LFC diet contained (w/w) 50.5% sucrose, 12.2% cornstarch, 5% corn oil and 5% cellulose, by weight [17] and was fed for a period of 3 weeks.

Plasma lipid and lipoprotein analysis

Levels of total plasma cholesterol and TAGs (without measuring free glycerol) were determined using commercially available enzymic kits nos. 236691 (Boehringer-Mannheim G.m.b.H., Mannheim, Germany) and 337-B (GPO-Trinder kit; Sigma, St. Louis, MO, U.S.A.). NEFA were measured enzymically with a NeFa-C kit (Wako Chemicals G.m.b.H., Neuss, Germany). TAGs and phospholipid in the isolated VLDL fractions were determined using a Boehringer-Mannheim enzymic assay kit (no. 701904) and an analytical kit (B; Wako Chemicals). Total cholesterol and non-esterified cholesterol in lipoprotein fractions were measured fluorimetrically ($\lambda_{\text{excitation}}$ 325 nm, $\lambda_{\text{emission}}$ 415 nm) in phosphate buffer, pH 7.4, using cholesterol oxidase and peroxidase (Boehringer-Mannheim), 0.5% Triton X-100 (Merck, Darmstadt, Germany), 20 mM cholic acid and 40 mg/l *p*-hydroxyphenylacetic acid (Sigma). Before measurements were made, cholesterol was extracted with ethanol/hexane/water (2:4:1, v/v). For total cholesterol extractions the ethanol solution contained 0.35 M KOH. Esterified cholesterol was calculated by the difference between total and non-esterified cholesterol.

Protein concentrations in lipoprotein fractions were determined by the method of Lowry et al. [18] with BSA as a standard.

Liver perfusion

Experiments were always started between 10.00 and 11.00 h. Fed mice were anaesthetized with an intraperitoneal injection of 0.5 ml/kg hypnorm (Janssen Pharmaceutica, Tilburg, The Netherlands) and 12.5 mg/kg midazolam (Roche Netherlands b.v., Mijdrecht, The Netherlands). After cannulation of the portal vein with an Abbocath-T cannule (26G*19 mm; Abbott Laboratories, Queenborough, Kent, U.K.), livers were isolated surgically and perfused (1.5 ml/min) *in vitro* in a recycling fashion as described previously for rats [19,20] with some minor modifications. Briefly, the liver and perfusate were maintained at 37 °C throughout the experiment. The recirculating perfusate (20–30 ml) was a RPMI buffer (RPMI 1640 medium, ICN Biomedicals, Costa Mesa, CA, U.S.A.), pH 7.5, containing 1 mM ascorbic acid (vitamin C), 5 mM glutathione and 2.5% (w/w) BSA. Oleate was added as an albumin complex to the perfusate at a final concentration of 1 mM in all experiments and was perfused throughout the remainder of the perfusion period. The perfusate was gassed with O₂/CO₂ (19:1). All perfused livers were still functionally viable by gross appearance after a period of 3 h perfusion. The 20–30 ml perfusate was collected at the end of the experiment and used to isolate nascent VLDL.

In one set of experiments, 500 i.u. of heparin (LEO Pharmaceutical Products, Weesp, the Netherlands) was injected into the spleen of each mouse 5 min before the beginning of the liver perfusion.

Lipoprotein isolation

VLDL fractions ($d < 1.006$) were isolated from pooled plasma of 15 mice per group by ultracentrifugation at 40000 rev./min in a SW-40 swingout rotor (Beckman, Geneva, Switzerland) for 18 h at 5 °C. Before isolation of nascent VLDL, the 20–30 ml of perfusate was concentrated to 10 ml using a

Centriprep. 30 (Amicon Division, Danvers, MA, U.S.A.) instrument. Each VLDL sample was dialysed against 0.1 M Tris buffer, pH 8.5, at 4 °C.

Apolipoprotein analysis of nascent VLDL

Nascent VLDL fractions (50 µg of TAGs) were analysed for apolipoprotein composition by using SDS/PAGE with 4–25% gradient gels. Proteins were either stained with Serva Blue R or transferred to nitrocellulose membranes (Schleicher and Schuell) followed by incubation with polyclonal rabbit antisera against mouse apoA1, apoE, apoC3 and apoC1 (kindly provided by Dr. K. Weisgraber, Gladstone Foundation Laboratories for Cardiovascular Disease, San Francisco, CA, U.S.A.). ¹²⁵I-labelled donkey anti-rabbit IgG (Amersham Corp.) was used as a secondary antibody. Detection of protein bands was performed by scanning the gels with a PhosphorImager (Molecular Dynamics).

Enrichment of apoE-deficient VLDL with apoE

Nascent apoE-deficient VLDL obtained by liver perfusion of apoE-deficient mice was incubated with increasing amounts of recombinant apoE3 as indicated (kindly provided by Dr. G. Siest, Centre du Médicament et Centre de Médecine Préventive, Nancy, France) at 37 °C for 1 h. Thereafter, apoE-enriched nascent VLDL was re-isolated by ultracentrifugation as described above to remove apoE that was not associated with nascent VLDL.

The amounts of recombinant apoE associated with VLDL were measured by 'sandwich' ELISA [21]. Briefly, affinity-purified polyclonal goat anti-(human apoE) antibodies were used for coating the plates. After incubation with the lipoprotein fractions, affinity-purified polyclonal goat anti-(human apoE) antibodies conjugated with horseradish peroxidase were used as second antibodies. Detection was done by the immunoperoxidase procedure using tetramethylbenzidine as a substrate. Pooled plasma from healthy human subjects with known apoE level was used as a standard.

The amounts of endogenous mouse apoE on VLDL were measured by using SDS/PAGE with 4–25% gradient gels and Serva Blue R protein staining as described above. Samples with known human apoE concentrations (measured by the sandwich ELISA) were used as a standard.

Assay of lipolysis with LPL in solution

Lipolysis assays were performed at 37 °C in a 0.1 M Tris buffer, pH 8.5, for 10 min with bovine LPL (0.2 unit; Sigma) in the presence of 2% (w/v) albumin (essentially NEFA-free). The reaction was stopped by the addition of 50 mM KH₂PO₄/0.1% Triton X-100, pH 6.9, and the mixture placed on ice. To obtain a zero-time control, the reaction was prevented by adding Triton prior to the addition of LPL and the mixture placed on ice. NEFA were measured by using the enzymic colorimetric method as described above. The rate of NEFA release by 0.2 unit of LPL was linear for the 10 min period used in this assay. The assay was performed using three different concentrations of VLDL TAGs ($d < 1.006$) in the range 0.1–0.3 mmol/l with duplication of NEFA determination.

Assay of lipolysis with HSPG-bound LPL

Wells of a 96-well microtitre plate (Greiner G.m.b.H., Frickenhausen, Germany) were incubated with 0.1 µg of HSPG (Sigma) in 75 µl of PBS for 18 h at 4 °C. Aspecific binding sites

were blocked by incubation with 1 % (w/v) BSA in 100 μ l of PBS for 1 h at 37 °C. Subsequently, wells were incubated with 1 unit of LPL (Sigma) in 75 μ l Tris/glycerol buffer [0.1 M Tris/20 % (v/v) glycerol, pH 8.5] for 1 h at 4 °C. Unbound LPL was removed by washing the plates three times with 0.1 M Tris. The reaction was initiated by the addition of a VLDL TAG sample to the well and placing the plate in a shaking incubator at 37 °C. The reaction was stopped after 20 min by the addition of 1 % (v/v) Triton X-100 in 0.1 M Tris, vortex-mixing and cooling on ice. Under these assay conditions, the rate of NEFA released by LPL bound to HSPG was linear for 20 min. The assay was performed on three different concentrations of VLDL TAGs ($d < 1.006$) in the range of 0.1–0.3 mmol/l and NEFA concentrations were measured enzymically in duplicate as described above.

RESULTS

Plasma lipid levels and VLDL lipid composition

As shown in Table 1, apoE-deficient mice have elevated levels of plasma cholesterol and TAGs as compared with wild-type mice on a regular chow diet. However, with mice on a chow diet, apoE-deficient plasma VLDL ($d < 1.006$), as isolated by ultracentrifugation, was relatively poor in TAGs and strongly enriched in cholesterol ester as compared with plasma VLDL isolated from wild-type mice (Table 2).

The low TAGs level in apoE-deficient VLDL could be a consequence of its prolonged residence time and concomitant lipolysis in the circulation. In order to obtain apoE-deficient VLDL with a higher TAG content, mice were fed an LFC sucrose-based diet which is known to enhance hepatic VLDL TAG production [16]. On this diet, plasma TAG levels were slightly elevated in apoE-deficient mice as compared with the chow diet, whereas plasma lipid levels were not affected in wild-type mice (Table 1). However, as shown in Table 2, with mice on the LFC diet, apoE-deficient plasma VLDL was still poor in TAGs as compared with plasma VLDL from wild-type mice. Furthermore, it was found that apoE-deficient plasma VLDL contained relatively high levels of NEFA, a phenomenon that was not observed for plasma VLDL of normal mice. As measured enzymically, the molar TAG/NEFA ratio of apoE-deficient plasma VLDL was strongly decreased as compared with the molar TAG/NEFA ratio of wild-type plasma VLDL (0.7 ± 0.1 versus 13.4 ± 0.1 respectively). This low TAG/NEFA ratio of apoE-deficient plasma VLDL made it impossible to adequately monitor NEFA release as a result of lipolysis by LPL. Thus both

Table 2 Relative lipid composition of plasma VLDL

Mice were fed a normal Chow diet or an LFC diet for a period of 3 weeks. VLDL ($\rho < 1.006$ g/ml) was isolated by ultracentrifugation from pooled plasma of at least 15 wild-type mice and ten apoE-deficient mice. The lipid levels were measured enzymically as described in the Methods section and are the means $\pm$ S.D. for three different VLDL pool samples. Abbreviations: FC, free cholesterol; CE, cholesterol ester; PL, phospholipids. * $P < 0.05$, indicating the difference between wild-type and apoE-deficient VLDL using non-parametric Mann–Whitney tests.

Plasma VLDL	Diet	Composition [% (w/w) of total lipid]			
		TAGs	FC	CE	PL
Wild type	Chow	51.6 ± 4.4	16.1 ± 2.2	10.8 ± 5.3	21.5 ± 3.4
ApoE-deficient	Chow	$6.7 \pm 0.8^*$	$13.0 \pm 0.5^*$	$64.1 \pm 1.8^*$	16.3 ± 0.9
Wild type	LFC	65.2 ± 7.2	4.4 ± 1.1	10.5 ± 3.5	19.9 ± 3.8
ApoE-deficient	LFC	$9.2 \pm 1.2^*$	$18.0 \pm 3.6^*$	$47.5 \pm 10.9^*$	25.3 ± 8.8

the relatively low TAG content as well as its association with high NEFA levels make apoE-deficient plasma VLDL less suitable as a substrate for investigating LPL-mediated lipolysis.

Production and characterization of nascent VLDL

Previous studies have shown that perfused rat livers secrete nascent VLDL that is rich in TAG, owing to the fact that, in contrast with plasma VLDL, nascent VLDL has not been exposed to LPL in the circulation [19]. Thus, in order to obtain TAGs-rich VLDL from apoE-deficient mice, we decided to isolate nascent VLDL obtained by liver perfusions with mice.

Our first objective was to characterize the properties of nascent VLDL as produced by mouse livers in wild-type mice. The rate of VLDL TAG production by mouse livers was constant during 3 h of perfusion (results not shown), indicating that the liver is functionally viable during 3 h of perfusion. Furthermore, wild-type mice were fed either a normal chow diet or a sucrose-rich (LFC) diet for a period of 3 weeks prior to the liver-perfusion experiments. After 3 h of perfusion, livers of mice fed the LFC diet had secreted three times as much VLDL TAGs in the perfusate as livers from chow-fed mice (300 ± 86 versus 109 ± 22 μ g of TAG/g of wet liver). To investigate whether the presence of hepatic lipase in the liver would affect nascent VLDL

Table 1 Plasma lipid levels in apoE-deficient and wild-type mice

Total cholesterol (TC) and TAGs were measured in plasma of wild-type mice and apoE-deficient mice fed a regular Chow diet or an LFC sucrose-based diet for a period of 3 weeks. Values are means $\pm$ S.D. for ten mice per group.

Mice	Diet ...	Concentration (mmol/litre)			
		Chow		LFC	
		TC	TAGs	TC	TAGs
Wild type		2.4 ± 0.4	0.5 ± 0.2	2.8 ± 0.2	0.3 ± 0.1
ApoE-deficient		14.3 ± 3.9	0.9 ± 0.4	11.7 ± 2.2	1.7 ± 0.5

Table 3 Relative lipid composition of nascent VLDL

Nascent VLDL ($\rho < 1.006$ g/ml) was isolated by ultracentrifugation from liver perfusates of wild-type mice and apoE-deficient mice fed the LFC diet. The lipid composition of nascent VLDL was measured enzymically as described in the Methods section and values are means $\pm$ S.D. for three individual nascent VLDL samples. Abbreviations: FC, free cholesterol; CE, cholesterol ester; PL, phospholipids. * $P < 0.05$, indicating the difference between wild-type and apoE-deficient VLDL using non-parametric Mann–Whitney tests.

Nascent VLDL	Composition [% (w/w) of total lipid]			
	TG	FC	CE	PL
Wild type	73.8 ± 1.2	3.1 ± 1.0	3.4 ± 1.3	19.7 ± 0.8
ApoE-deficient	77.9 ± 3.8	2.5 ± 0.8	6.4 ± 2.0	$13.2 \pm 3.6^*$

production, a heparin injection was given to the spleen prior to the beginning of the liver perfusions. Previous studies have shown that most of the hepatic lipase activity on liver cell surfaces can be displaced by heparin injection [22]. Under LFC-fed conditions, the output of VLDL TAGs of livers from mice pre-injected with heparin was not significantly different from that from untreated livers (228 ± 45 versus 300 ± 86 μg of TAG/g of wet liver). Thus these data indicate that the hepatic VLDL TAG production is not affected by hepatic lipase in the liver.

Our next step was to isolate nascent VLDL from perfused livers of apoE-deficient mice. The apoE-deficient mice had been fed the LFC diet and livers were perfused for a period of 3 h. Strikingly, nascent VLDL of apoE-deficient mice was similar in lipid composition to nascent VLDL isolated from wild-type mice (Table 3). In contrast with plasma apoE-deficient VLDL, nascent VLDL of both apoE-deficient and wild-type mice contained low amounts of NEFA relative to TAGs (TAG/NEFA molar ratio of 20.5 ± 0.1 and 21.0 ± 0.1 respectively). In addition, both apoE-deficient and wild-type nascent VLDL were more rich in TAGs as compared with VLDL isolated from plasma of these mice (compare Table 3 with Table 2), although the difference in TAG content between plasma and nascent wild-type VLDL was not statistically significant.

The apolipoprotein composition of apoE-deficient- and wild-type-mouse nascent VLDL was analysed by SDS/PAGE. As expected, in VLDL isolated from the liver perfusate of apoE-deficient mice, apoE was completely absent, whereas the amount of apoA4 was increased as compared with that in wild-type-mouse nascent VLDL (Figure 1). The relative amount of endogenous mouse apoE on nascent wild-type-mouse VLDL was calculated and is depicted in Table 4.

Effect of apoE on the LPL-mediated lipolysis of VLDL-TAGs

A separate series of lipolysis experiments showed that the rate of NEFA release from wild-type-mouse nascent VLDL-TAGs was linear for 10 min at an LPL level of 0.2 unit. The apparent K_m and V_{max} values were calculated by Lineweaver–Burk analysis and were 0.22 ± 0.05 mmol/litre VLDL TAG and 80 ± 50 μmol of NEFA/min per litre respectively. All lipolysis experiments were further performed with substrate concentrations in the calculated K_m value range, varying from 0.1 to 0.3 mmol/litre VLDL-TAGs.

To investigate the effect of apoE-deficiency on the *in vitro* LPL-mediated hydrolysis of TAGs, nascent apoE-deficient VLDL and wild-type VLDL were lipolysed with LPL in solution. The NEFA release from nascent wild-type VLDL upon treatment with LPL was similar to the NEFA release from nascent apoE-deficient VLDL (Table 4).

To study directly the effect of apoE on the *in vitro* LPL-mediated hydrolysis of TAGs, apoE-deficient nascent VLDL was enriched with apoE. Therefore nascent apoE-deficient VLDL was incubated with various amounts of recombinant apoE for 1 h at 37 °C. Thereafter, apoE-enriched nascent VLDL was re-isolated by density-gradient ultracentrifugation to remove free apoE and the VLDL apoE content was measured by sandwich-ELISA analysis (Table 4). It was determined by SDS/PAGE and Western-blot analysis that enrichment of the apoE-deficient nascent VLDL with apoE decreased the amounts of apoA4 and apoA1 on the particle (results not shown). ApoE-enriched nascent VLDL was tested as a substrate for LPL using two different lipolysis assays. First, to 0.2 unit of LPL in solution, different concentrations of VLDL-TAGs were added and the release of NEFA was measured. As depicted in Table 4, increasing

Figure 1 Apolipoprotein composition of nascent VLDL

Nascent VLDL ($\rho < 1.006$ g/ml) was isolated by ultracentrifugation from liver perfusates of wild-type mice (WT) (lane 1) and apoE-deficient mice (E0) (lane 2) fed the LFC diet. VLDL TAGs (50 μg of TAGs) was subjected to SDS/PAGE (4–25% gradient gels) and the VLDL proteins were stained with Serva Blue R.

Table 4 Effect of increasing amounts of apoE on the hydrolysis of VLDL TAGs with LPL in solution and bound to HSPG

Nascent VLDL ($d < 1.006$) was obtained through wild-type and apoE-deficient mouse liver perfusions. ApoE-deficient nascent VLDL was gradually enriched with the indicated amounts of recombinant apoE upon incubation at 37 °C for 1 h. The VLDL-associated apoE levels were measured using sandwich ELISA or SDS/PAGE (the amount of apoE relative to the amount of TAGs is indicated). Both LPL in solution and LPL bound to HSPG were used as described in the Methods section. Values are means $\pm$ S.D. with NEFA release at a TAG concentration of 0.2 mmol/litre. Since three independent experiments are combined, all values are expressed as a percentage of control values (no addition of apoE). The mean NEFA release of apoE-deficient nascent VLDL was 29.7 ± 2.1 μmol of NEFA/min per litre with LPL in solution and 8.4 ± 0.5 μmol FFA/min per litre with LPL bound to HSPG at 0.2 mmol/litre of the respective VLDL TAG samples. Similar results were obtained with VLDL TAG concentrations at 0.1 and 0.3 mmol/litre. * $P < 0.05$, indicating the difference between apoE-deficient VLDL without and with the addition of apoE, using non-parametric Mann–Whitney tests.

Nascent VLDL	ApoE ($\mu\text{g}/\text{mg}$ of VLDL TAGs)	FFA release (% of apoE-deficient VLDL)	
		LPL in solution	HSPG–LPL
ApoE-deficient	0	100.0 $\pm$ 7.0	100.0 $\pm$ 7.4
Wild-type	1.3	92.4 $\pm$ 6.3	—
ApoE-deficient + apoE	2.7	63.1 $\pm$ 6.3*	72.3 $\pm$ 3.6*
	11.9	54.5 $\pm$ 5.8*	—
	29.6	20.8 $\pm$ 1.8*	56.6 $\pm$ 1.7*

amounts of apoE on nascent apoE-deficient VLDL resulted in a gradual decrease in NEFA release.

Secondly, since *in vivo* LPL is bound to HSPG at the surface of endothelial cells [23], we also performed an *in vitro* lipolysis assay using LPL bound to HSPG in order to preserve the interaction between the lipoprotein and the HSPG–LPL complex. Increasing amounts of apoE on nascent VLDL particles inhibited the hydrolysis of TAGs as mediated by the HSPG–LPL complex, although to a lesser extent as compared with the assay with LPL in solution (Table 4).

DISCUSSION

In the present study we assessed directly whether apoE would effect TAGs hydrolysis by using apoE-deficient VLDL. ApoE-deficient VLDL as isolated from pooled plasma of apoE-deficient mice was not suitable to investigate as a substrate for the LPL-mediated lipolysis owing to its relatively low TAG content, as well as its association with high NEFA levels. Therefore, we decided to use the nascent VLDL obtained through mouse liver perfusions. Interestingly, it was demonstrated that the livers of apoE-deficient mice secreted nascent VLDL that was rich in TAGs and very similar in lipid composition as compared with the nascent VLDL secreted by livers of wild-type mice. Furthermore, the LPL-mediated NEFA releases from nascent apoE-deficient VLDL and nascent wild-type VLDL were not significantly different (Table 4), indicating that, in the total absence of apoE, TAG-rich lipoproteins are not more readily lipolysed. These results strongly suggest that the relatively low TAG content of VLDL remnant particles in plasma of apoE-deficient mice results from their longer residence time and concomitant exposure to LPL in the circulation, as a consequence of the diminished apoE-dependent VLDL clearance rate.

Whereas the relatively low amount of endogenous mouse apoE on nascent wild-type VLDL did not affect TAG hydrolysis, larger amounts of apoE on the nascent VLDL particle were shown to dose-dependently inhibit TAGs hydrolysis by LPL in solution (Table 4). Furthermore, it was demonstrated that enrichment of the nascent apoE-deficient particle with apoE decreased the amounts of both apoA1 and apoA4 on the particle. Therefore the possibility that a decrease in apoA4 and apoA1 on nascent VLDL caused impaired VLDL lipolysis cannot be excluded. However, previous studies have not reported on an impaired VLDL lipolysis in apoA1-deficient mice [24,25]. Furthermore, increasing amounts of apoA1 on artificial TAG emulsions did not alter its suitability as a substrate for LPL [9].

Increasing amounts of apoE on nascent VLDL were also shown to inhibit TAG hydrolysis when lipolysis experiments were carried out with LPL bound to HSPG (Table 4). Previously, Clark and Quarfordt [26] reported that the addition of apoE to TAG-rich emulsions resulted in a stimulation rather than an inhibition of lipolysis using LPL immobilized to heparin. It was suggested that this stimulation was due to an increased binding affinity of these emulsions to the column mediated by its high content of apoE (175 µg of TAGs/mg). These results suggest that, in the present study, the impaired TAG hydrolysis, as observed after the addition of relatively low amounts of apoE (3–30 µg of TAGs/mg) to nascent VLDL, is not overwhelmed by an increased binding affinity of the particle to HSPG. Presumably, apoE directly affects the interaction of LPL with VLDL TAGs, which would also be in line with our results obtained with LPL in the solution assay (Table 4). Similar findings have previously been reported by other groups as well [8,9]. It was shown that a synthetic peptide (residues 139–153), corresponding to the arginine/lysine-rich receptor-binding domain of apoE, inhibited LPL activity [8]. More evidence that this domain was responsible for the inhibitory action of apoE on LPL was provided by the observation that the inhibitory effect of apoE on artificial-emulsion-TAG hydrolysis could be abolished by the modification of arginine residues in apoE through incubation with cyclohexane-1,2-dione [9].

The question whether the inhibition of LPL by apoE is of physiological importance in plasma TAG metabolism remains to be answered. It can be hypothesized that, during LPL-mediated lipolysis, gradual enrichment of VLDL with apoE progressively inhibits lipolysis and directs the partially lipolysed VLDL

particles to receptor-mediated uptake in the liver. From this point of view the presence of apoE on the lipoprotein particle will not be likely to cause elevated levels of plasma TAGs, since the inhibitory action of apoE on VLDL lipolysis will be overwhelmed by the role of normal apoE in the mediation of particle uptake by the liver. However, experimental evidence has also been provided by our group that the inhibitory action of apoE on TAGs hydrolysis may contribute to elevated plasma TAGs levels (K. Willems van Dijk, van Vlijmen, B. J. M., M. H. Hofker and L. M. Havekes, unpublished work). Preliminary results show that overexpression of human apoE in apoE-deficient mice decreases plasma lipid levels at lower levels of apoE expression, but strongly elevates plasma TAGs levels at higher levels of apoE expression. These results suggest that the presence of human apoE on VLDL from apoE-deficient mice may also hamper the LPL-mediated lipolysis of these particles *in vivo* and can elevate plasma TAGs levels, providing that the amount of apoE on the lipoprotein particle is significantly increased. Furthermore, it has been reported that TAG-rich lipoproteins of subjects heterozygous for *APOE**2 (Lys¹⁴⁶ → Gln) contained a relatively high amount of apoE per particle concomitant with an impaired conversion of VLDL into intermediate-density lipoprotein [27]. More recently, we reported that overexpression of human *APOE**3Leiden [21] and human *APOE*2 (Arg¹⁵⁸ → Cys) [28] caused elevated levels of plasma TAGs in transgenic mice, well beyond that attributable to an impaired receptor-mediated clearance of lipoprotein particles. *In vivo*-turnover studies revealed an impaired lipolysis of VLDL TAGs in human *APOE**3Leiden transgenic mice. However, since the NEFA release from *APOE**3Leiden VLDL did not differ from control VLDL using the LPL in solution assay, it was suggested that a possible decreased binding affinity of *APOE**3Leiden VLDL to HSPG would account for the impaired *in vivo* lipolysis as observed in *APOE**3Leiden transgenic mice [21]. Thus apoE variants may affect lipolysis *in vivo* by modulating the binding of the lipoprotein to the LPL–HSPG complex, in addition to the direct inhibition of LPL activity shown for normal apoE in the present study.

In summary, *in vitro* enrichment of the VLDL particle with apoE effectively inhibits the LPL-mediated hydrolysis of TAGs which is, at least partly, independent of its affinity for proteoglycans. Thus, whereas apoE on VLDL facilitates its uptake by hepatic receptors, it also modulates VLDL lipolysis, implying a dual role for apoE in the clearance of TAGs-rich lipoproteins.

This research was supported by the Netherlands Heart Foundation, the Netherlands Foundation of Scientific Research and by Grants BIOMED-2 from the specific RTD Program of the European Commission (projects 901-04-092, 903-39-117 and BMH4-CT96-0898, respectively). M.H.H. is an Established Investigator of the Netherlands Heart Foundation.

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