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Differential effects of apolipoprotein E isoforms on lipolysis of very low-density lipoprotein triglycerides

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Abstract

Apolipoprotein (apo) E plays a key role in lipoprotein metabolism and has been proposed to modulate triglyceride (TG) lipolysis. However, no systematic investigation on lipolysis using all 3 isoforms of apoE has been performed. To clarify the role of common human apoE isoforms in the lipolysis of very low-density lipoprotein (VLDL) TGs, we overexpressed human apoE isoforms in apoE and low-density lipoprotein receptor–deficient mice using adenoviral-mediated gene transfer and used VLDL particles obtained from these mice for in vitro lipolysis assay. Overexpression of apoE, regardless of its isoforms, increased the TG content of VLDL in mice in vivo. In vitro analysis of the effect of apoE on lipolysis revealed that irrespective of its isoforms, apoE did inhibit TG lipolysis at every concentration of apoE examined, and this inhibitory effect became more pronounced as the apoE content of VLDL increased. No difference was observed in TG lipolysis activity among isoforms at low apoE/TG ratio; however, intermediate ratios of apoE/TG, which reflect physiologic VLDL apoE/TG ratios, demonstrated a significantly greater level of lipolysis inhibition in apoE2, but less so in apoE4 compared with other isoforms. This differential effect by apoE isoforms on lipolysis was attenuated at higher apoE/TG ratios; nevertheless, apoE2 still inhibited lipolysis significantly more than did apoE4. Enrichment of VLDL with apoE decreased both the apoC contents and apoC-II/C-III ratios of VLDL, contributing, at least in part, to the inhibitory function of apoE on lipolysis. The present study clarifies the differential lipolysis-modulating effect of apoE isoforms, which would help explain the difference in pre- and postprandial TG levels among humans carrying different apoE isoforms.

1. Introduction

Dyslipidemia is a major risk factor for atherosclerotic diseases. Epidemiologic and interventional studies have shown that low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels have strong positive and negative respective associations with coronary heart disease [1-3]. Several studies have suggested that increased plasma triglycerides (TGs) level is also a risk factor for coronary heart disease [4]. Furthermore, recent studies have proposed that postprandial dyslipidemia or hypertriglyceridemia accelerates atherosclerosis through several mechanisms including the accumulation of atherogenic remnant lipoprotein particles and an increase in the number of small dense low-density lipoprotein (LDL) particles [5].

Lipoprotein lipase (LPL), anchored on heparan sulfate proteoglycan (HSPG) of the vessel walls, plays a key role in the catabolism of lipoprotein core TGs using apolipoprotein (apo) C-II as an activator or a coenzyme [6-8]. A deficiency in these proteins causes type I hyperlipoproteinemia, where the plasma TG level usually exceeds 1000 mg/dL.

Apolipoprotein E is a ligand for several lipoprotein receptors and plays a key role in the receptor-mediated uptake of apoB-containing lipoproteins [9]. In addition to its well-established function, apoE is proposed to modulate TG lipolysis [10]. Several lines of evidence suggest that apoE inhibits the lipolysis of very low-density lipoprotein (VLDL) TGs [11-13], whereas other investigators have shown an increased lipolytic activity by apoE [14,15].

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There are 3 major apoE isoforms in humans: apoE2, E3, and E4. Epidemiologic studies have shown that this polymorphism is associated with interindividual variations in plasma lipid levels as well as susceptibility to atherosclerosis [10,16]. Apolipoprotein E4 is associated with higher plasma total cholesterol (TC) and LDL-C levels and higher apoB levels compared with apoE3. In addition, apoE4 is proposed to be an independent risk factor for coronary heart disease regardless of its effect on plasma cholesterol levels [17-19]. On the other hand, homozygotes for apoE2, insofar as they do not develop type III hyperlipoproteinemia, show lower plasma TC and LDL-C levels, lower apoB levels, and higher plasma TG levels compared with those of apoE3. The increase in postprandial plasma TG levels is prominent in homozygotes of apoE2 compared with those of apoE3 and apoE4 [20,21]. Furthermore, apoE2 is proposed to reduce the risk of atherosclerotic diseases. However, homozygotes for apoE2 who develop type III hyperlipidemia due to secondary precipitating factors are susceptible to atherosclerotic diseases [22,23].

The plasma TG level is determined by 3 steps in the lipoprotein metabolic pathway: (1) production of VLDL particles from the liver, (2) lipolysis of lipoprotein core TGs by lipase in the systemic circulation, and (3) uptake of TG-rich lipoprotein particles through the lipoprotein receptors. Previous studies have extensively examined the differential roles of apoE isoforms in the lipoprotein production in the liver [24,25] and their binding capacity to lipoprotein receptors [9,26]; however, no systematic investigation on lipolysis using all 3 isoforms has been performed. To clarify the role of apoE isoforms in the lipolysis of VLDL TGs, we overexpressed human apoE isoforms in mice deficient in both apoE and LDL receptor (LDLR) genes using adenoviral-mediated gene transfer and thus used VLDL particles obtained for the in vitro lipolysis assay. The present study provides the first comprehensive data demonstrating the inhibitory effect of all 3 apoE isoforms in the lipolysis of VLDL TGs and the isoforms' differential lipolysis-modulating effects.

2. Experimental design and methods

2.1. Materials

ApoE/LDLR double knockout mice were raised in the Animal Center for Biomedical Research at the University of Tokyo. They were fed with a normal chow. Second-generation adenoviral vectors AdE2, AdE3, and AdE4 [25], encoding apoE2, E3, and E4 isoform complementary DNA, respectively, were used to express human apoE isoforms. Bovine milk LPL was purchased from Sigma-Aldrich (St Louis, MO).

2.2. Preparation of plasma VLDL

Three groups of 12- to 16-week-old apoE/LDLR double knockout mice were injected intravenously with 6×10^9 plaque-forming units of AdE2, AdE3, or AdE4 through the tail vein; each group contained 2 mice. Three days after the injection of adenoviruses, the mice were placed on a lipid-free diet for 4 hours to minimize the contamination of chylomicron, and their blood was obtained from the retro-orbital plexus at a volume of 0.5 to 0.7 mL per mouse. Blood sampling was also performed on mice not injected with an adenovirus to extract VLDL free of apoE protein (apoE-null VLDL). Plasma was immediately separated by centrifugation and diluted with saline (d = 1.006). Diluted plasma samples were subjected to ultracentrifugation at 40 000 rpm for 3 hours using RPL-42T rotor (Hitachi, Tokyo, Japan) [27], a rotor equivalent to the LP 42 Ti rotor (Beckman Coulter, Fullerton, CA) [28,29], after which the upper white layer was collected as VLDL. Extracted VLDLs from 2 mice were pooled together for each isoform, and TC and TG concentrations were measured enzymatically using Cholesterol C-test Wako kit and Triglyceride G-test Wako kit (Wako Pure Chemicals Industries, Osaka, Japan), respectively. Apolipoprotein E concentration in VLDL was measured using the immunoturbidimetric method with ApoE auto N (Daiichi) reagents (Daiichi Pure Chemicals, Tokyo, Japan).

2.3. In vitro lipolysis assay

Very low-density lipoprotein containing each apoE isoform was gently mixed with apoE-null VLDL and incubated for 2 hours at 37°C to equalize the concentration ratio of apoE/TG among VLDLs carrying each apoE isoform. Very low-density lipoprotein apoE was adjusted at 10, 20, 30, 50, and 70 μg/mg TG, respectively. Adjusted VLDL was dissolved in a buffer containing 2% bovine serum albumin (BSA) and 100 mmol/L Tris, pH 8.5, to set final TG concentrations of each sample at 30 mg/dL.

Lipolysis reaction was initiated by the addition of 1.25 U of bovine LPL. Fifteen minutes later, the reaction was terminated by adding stop solution containing 50 mmol/L KH_2PO_4 and 0.1% Triton X-100. As a control reaction, LPL and stop solution were added simultaneously to the samples. Nonesterified fatty acid concentrations of all samples were measured with NEFA-C kit WAKO (Wako Pure Chemicals Industries). Fatty acid release by lipolysis reaction was calculated by dividing the increment of fatty acid concentrations in each sample by reaction time. Each reaction was performed in sextuplets, and the lipolysis rate was expressed as a percentage, setting the fatty acid release of apoE-null VLDL sample at 100%.

2.4. Isoelectric focusing of apoC-II and apoC-III

After equalization of the VLDL-apoE/TG ratio, VLDL samples were diluted in d = 1.006 saline and ultracentrifuged again at 40 000 rpm for 2 hours. The upper layer was collected as VLDL and delipidated by adding an ethanol-ether (3:1) mixture. Equal amounts of VLDL (corresponding to 18 μg TG) was dissolved in 100 μL of isoelectric focusing (IEF) sample buffer (100 mmol/L Tris, 6 mol/L urea, 1% wt/vol decyl sodium sulfate, 5% vol/vol 2-mercaptoethanol, and

1%wt/vol glycerol). Twenty microliters of each sample was electrophoresed on a 13% polyacrylamide IEF gel (pH 4-6.5) at 250 V overnight at 4°C and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) for 1 hour at room temperature and then incubated with antirabbit anti-apoC antibody (Biodesign International, Saco, ME) for 16 hours at 4°C, followed by incubation with horseradish peroxidase–conjugated goat antirabbit immunoglobulin G as a secondary antibody. An enhanced chemiluminescence ECL Plus kit (Amersham Biosciences, Piscataway, NJ) was used for the detection method. Images of the membrane were captured using computerized CCD camera LAS-1000 system (Fuji Photo Film, Tokyo, Japan) and analyzed by NIH Image software.

2.5. Statistical analysis

All data are presented as mean ± SEM. Statistical comparisons were made by 1-way analysis of variance using Prism Version 4 software (GraphPad Software, San Diego, CA). A 95% confidence level was deemed statistically significant.

3. Results

3.1. Lipid composition of VLDL after expression of each apoE isoform

Table 1 shows the lipids and apoE composition of VLDL purified and pooled from the apoE/LDLR double knockout mice injected with apoE adenoviruses. The VLDL TG/TC ratio increased with the expression of all 3 apoE isoforms. The apoE/TG ratio of VLDL was similar among all 3 isoforms (E2, 111.1 μg/mg TG; E3, 104.1 μg/mg TG; E4, 70.4 μg/mg TG).

3.2. Effect of apoE isoforms on VLDL lipolysis

The VLDL apoE concentration was adjusted to 10, 20, 30, 50, and 70 μg/mg TG by incubation with apoE-null VLDL and subjected to in vitro lipolysis assay. The VLDL TC/TG ratios after adjusting the apoE/TG ratio are shown in Table 2. The VLDL TC/TG ratio decreased in all isoforms as the apoE/TG ratio increased. Comparing all 3 apoE isoforms at equal apoE/TG ratios revealed that the VLDL TC/TG ratio was highest in apoE2-VLDL and lowest in apoE4-VLDL.

Table 1
Composition of VLDL purified from apoE/LDLR double knockout mice injected with apoE adenoviruses

Component	Adenovirus			
	(−)	AdE2	AdE3	AdE4
apoE	0	67.5	69.5	115.4
TG	257.8	607.6	667.6	1639.6
TC	984.5	781.8	589.7	891.1
TC/TG ratio	3.82	1.29	0.88	0.54
ApoE/TG ratio	0	0.111	0.104	0.070

Data are expressed in mg/dL. (−), without injection of adenovirus.

Table 2
VLDL TC/TG ratios after adjustment of apoE/TG ratios

	apoE/TG (μg/mg)				
	10	20	30	50	70
apoE2-VLDL	3.59	3.36	3.13	2.68	2.22
apoE3-VLDL	3.54	3.25	2.97	2.41	1.84
apoE4-VLDL	3.35	2.89	2.42	1.49	0.56

Very low-density lipoprotein containing each apoE isoform was gently mixed with apoE-null VLDL and incubated for 2 hours at 37°C to adjust the final apoE concentrations to 10, 20, 30, 50, and 70 μg/mg TG. Total cholesterol and triglyceride concentrations were measured in all samples for confirmation, followed by calculation of the TC/TG ratio.

The lipolysis rates, calculated by dividing released NEFA content by reaction time, are shown in Fig. 1. Because the rate of NEFA release was linear for at least 20 minutes, the lipolysis rate was determined from NEFA released in the initial 15 minutes. The lipolysis rates analyzed using apoE-null VLDL as a substrate were defined as 100%. As shown in Fig. 1A, lipolysis rates decreased significantly in all 3 apoE isoforms as the VLDL apoE/TG ratio increased. However, there was a characteristic difference in the pattern of decline among apoE isoforms. When the apoE/TG ratio was as low as 10, there was no difference in lipolysis rates among the 3 isoforms (E2, 91.8% ± 1.5%; E3, 95.5% ± 2.7%; E4, 89.6% ± 0.9%). When the apoE/TG ratios were increased to 20 (E2, 75.6% ± 0.2%; E3, 81.3% ± 1.8%; E4, 95.9% ± 0.5%) and 30 (E2, 64.4% ± 1.1%; E3, 76.1% ± 1.3%; E4, 90.6% ± 0.8%), the inhibition of lipolysis was greatest in apoE2 ($P < .01$ vs apoE3, $P < .001$ vs apoE4) and least in apoE4 ($P < .01$ vs apoE3); as for apoE4, there was no difference in lipolysis rates while apoE/TG ratios ranged between 10 and 30. However, when apoE/TG ratios were increased to 50 (E2, 55.1% ± 1.5%; E3, 58.3% ± 3.3%; E4, 61.0% ± 1.2%) and as high as 70 (E2, 48.1% ± 0.3%; E3, 50.6% ± 0.2%; E4, 55.0% ± 0.3%), the isoform-dependent differential effect on lipolysis was reduced. Nevertheless, apoE2 still demonstrated significantly greater inhibitory activity than apoE4 ($P < .05$).

3.3. Effect of apoE isoforms on VLDL apoC-II and apoC-III

We next examined whether an increase in apoE content within VLDL modified the apoC-II and/or apoC-III contents of VLDL particles. Proteins were purified from VLDL samples whose apoE/TG ratios were adjusted to 0, 30, and 70, and were subjected to IEF gel electrophoresis followed by Western blot analysis. As shown in Fig. 2A, in all 3 isoforms, both apoC-II and apoC-III contents decreased as the apoE/TG ratios increased. The apoC-II/C-III ratio, which was analyzed using a densitometer, also decreased as the apoE/TG ratios increased in all 3 isoforms (Fig. 2B). The apoC-II/C-III ratio tended to be less in apoE4-VLDL compared with apoE2- and apoE3-VLDL.

4. Discussion

Apolipoprotein E exists on the surface of several lipoprotein classes, especially on TG-rich lipoproteins such as chylomicron and VLDL. Therefore, besides serving as a ligand for lipoprotein receptors and facilitating VLDL secretion from the liver, apoE is proposed to play an important role on the lipolysis of pre- and postprandial plasma TGs. Several studies have examined the role of apoE on TG lipolysis; however, their results were not conclusive [11-15]. In addition, it has been difficult to compare the effect on lipolysis rate of one apoE isoform to that of another using purified VLDL extracted from humans because the lipids and apoE composition of VLDL are different among apoE isoforms. Some investigators used artificial lipid emulsion to modify the lipid and apoE content [11,30]; however, their results need to be interpreted carefully because artificial VLDL is different from the native VLDL in several respects. Apolipoprotein E is an apolipoprotein that can exchange between lipoprotein particles. As reported previously [31], the dissociation constant of apoE bound to microemulsion becomes greater at 37°C compared with one at 4°C. Another report [30] showed that apoE exchanged from plasma lipoprotein particles to lipid emulsion particles by mixing these particles at 37°C. Thus, to minimize the effect caused by the discrepancies in VLDL composition, in the present study, we adjusted the ratio of apoE to TGs by mixing at 37°C VLDL particles containing apoE with apoE-null VLDL, which lacks only the apoE protein while possessing all other components of natural VLDL.

As shown in Table 1, overexpression of apoE, regardless of its isoforms, increased the TG content of VLDL in apoE/

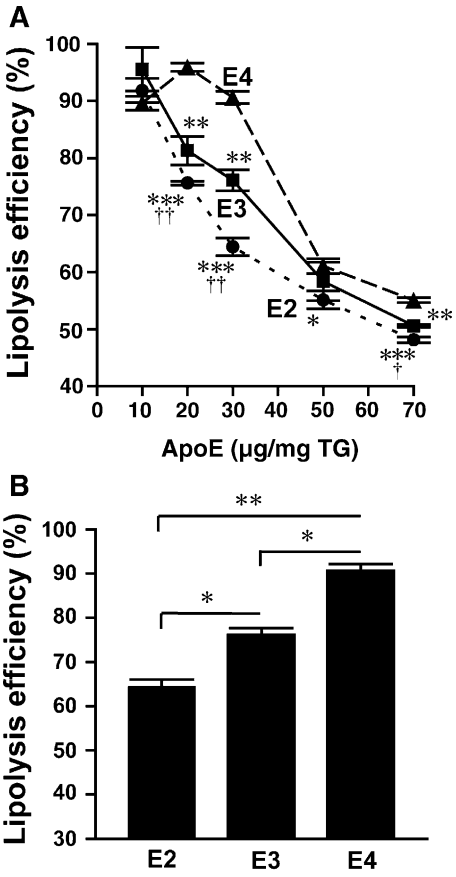

Fig. 1. A, Effect of apoE isoform on VLDL lipolysis. Lipolysis was initiated by adding 1.25 U of LPL to adjusted VLDL at a final triglyceride concentration of 30 mg/dL and terminated 15 minutes later by adding stop solution. Control reactions were performed by adding LPL and stop solution simultaneously. Nonesterified fatty acid concentrations of all samples were measured, and fatty acid release was determined by dividing the difference in fatty acid concentrations by reaction time. Lipolysis rate was calculated by setting the fatty acid release of apoE-null VLDL at 100%. The closed circles, closed squares, and closed triangles indicate apoE2, apoE3, and apoE4, respectively. Data are mean ± SEM (n = 6). $^{\dagger}P < .05$ vs apoE3; $^{\dagger\dagger}P < .01$ vs apoE3; $^{**}P < .01$ vs apoE4; $^{***}P < .001$ vs apoE4; $^{*}P < .05$ vs apoE4. B, Comparison of lipolysis rates among the 3 apoE isoforms at concentrations of apoE equivalent to physiologic plasma levels (apoE/TG ratio was 30 μg/mg). Data are mean ± SEM (n = 6). $^{*}P < .01$; $^{**}P < .001$.

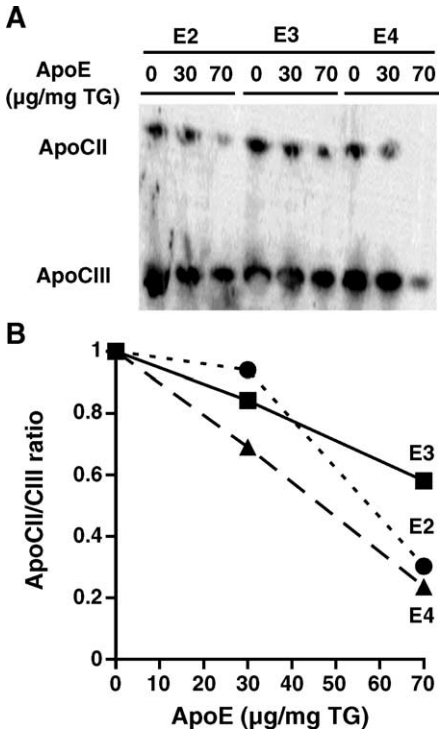

Fig. 2. A, Isoelectric focusing of mouse apoC-II and apoC-III. Very low-density lipoprotein samples containing 0, 30, and 70 μg/mg TG of apoE were dissolved in $d = 1.006$ saline and ultracentrifuged at 40 000 rpm for 3 hours. Very low-density lipoprotein, manifested as the upper white layer, was collected and delipidated with an ethanol-ether (3:1) mixture. Equal amounts of VLDL (containing 3.6 μg TG) were electrophoresed on a 13% polyacrylamide IEF gel (pH 4-6.5) at 250 V overnight at 4°C, followed by Western blot analysis using rabbit anti-mouse apoC antibody and horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G antibody as the primary and secondary antibodies, respectively. B, Very low-density lipoprotein apoC-II/C-III ratios for each apoE isoform. The image in A was analyzed with NIH Image software. ApoC-II/C-III ratio was calculated by dividing the intensity of the apoC-II signal by that of apoC-III, and the values were expressed with the apoC-II/C-III ratio of VLDL without apoE set at 1. The closed circles, closed squares, and closed triangles indicate apoE2, apoE3, and apoE4, respectively.

LDLR double knockout mice, implicating the inhibitory effect of apoE on VLDL TG lipolysis in vivo. A precise and systematic in vitro analysis of the effect of apoE on lipolysis using VLDL purified from these mice revealed that apoE inhibited lipolysis in TGs even at low concentrations, and this inhibitory effect became more pronounced as the apoE content increased (Fig. 1A). In addition, a comparison of this inhibitory effect among apoE isoforms revealed their differential inhibitory profiles. At low apoE/TG ratios, no difference was observed in the TG lipolysis activity among isoforms. However, intermediate ratios of apoE/TG reflecting the actual physiologic VLDL apoE/TG levels demonstrated a significantly greater level of lipolysis inhibition in apoE2, but less so in apoE4 compared with other isoforms (Fig. 1A and B).

One possible mechanism for the inhibitory effect of apoE on TG lipolysis is that an incremental change in apoE content within VLDL triggers displacement of the apoC-II and apoC-III—a potent activator and inhibitor of LPL, respectively—contents within VLDL. A previous study has confirmed that in apoC-III–overexpressing mice demonstrating a reduction in the lipolysis of TGs, levels of VLDL-apoC-II were reduced while those of VLDL–apoC-III increased [32]. As shown in Fig. 2, apoC-II, apoC-III, and apoC-II/C-III ratios decreased as apoE content increased in all 3 apoE isoforms. Therefore, alteration of apoE content on VLDL resulted in the changes in apoC contents of VLDL and affected, at least in part, the lipolysis of TGs.

However, these changes in apoC contents caused by the enrichment of VLDL with apoE do not completely explain the differential effect of apoE isoforms on lipolysis. Although the apoC-II content and apoC-II/C-III ratio tended to be less in apoE4-VLDL than in apoE2-VLDL, the inhibition of lipolysis by apoE2 was greater than that caused by apoE4, suggesting the existence of other mechanisms related to the isoform itself. One potential mechanism pertaining to the isoform itself may be the possibility that the interaction of apoE with apoB-containing lipoproteins might differ among 3 isoforms. Each apoE isoform is different from other isoforms by single arginine-cysteine interchanges at positions 112 and 158; apoE2 (112 Cys, 158 Cys), apoE3 (112 Cys, 158 Arg), and apoE4 (112 Arg, 158 Arg). Apolipoprotein E4, which possesses 2 arginines on both positions, is charged more positively than others. This property would reduce the affinity of the apoE4 protein to VLDL particles. Apolipoprotein E2, more rich in cysteine residues compared with the others, might bind tightly to VLDL particles through disulfide coupling, leading to a greater inhibitory effect on lipolysis. These mechanisms and hypotheses need to be clarified in future studies.

Some studies have proposed that as the TC content in VLDL increases, the lipolysis of TGs is inhibited [33]. As shown in Table 2, the TC/TG ratios in VLDL were higher in apoE2-VLDL than in apoE4-VLDL having the same apoE/TG ratios. These differences in TC/TG contents would, at least in part, result in the differential effects on lipolysis among the isoforms. However, in spite of decremental changes in TC/TG ratios with incremental changes in apoE/TG ratios in all 3 isoforms, the lipolysis rates decreased with every incremental change in apoE/TG ratios. Thus, we speculate that differences in TC content by itself do not explain the differential effects on lipolysis of the apoE isoforms.

de Man et al [34] proposed that the lipolysis of VLDL TGs is dependent on the apoE binding to HSPG. They demonstrated that apoE2 and apoE3-Leiden have less binding capability to HSPG compared with apoE3, leading to decreased lipolysis of VLDL carrying these apoE variants. We previously demonstrated that the binding ability of apoE2 to HSPG is reduced compared with that of apoE3 or apoE4 [35]. These evidences and our present study suggest that humans homozygous for apoE2 are susceptible to hypertriglyceridemia, a finding that correlates to actual clinical observations.

A possible limitation of our study is the utilization of bovine milk LPL. Previous studies have clarified that bovine milk LPL has almost similar molecular weight, lipase activity, and substrate specificity to human LPL, and is also activated by human apoC-II [36-38]. However, it remains to be seen whether soluble bovine milk lipase is similar in function to that of human LPL in the capillary endothelium, which is anchored on the HSPGs.

In summary, we demonstrated that apoE, regardless of its isoforms, inhibits VLDL-TG lipolysis even when its content within VLDL was below its physiologic counterpart. This inhibitory effect was at least partially due to the alteration of apoC contents within VLDL. In addition, we have for the first time demonstrated that apoE2 has the most and apoE4 the least inhibitory effect on lipolysis under physiologically sound apoE levels within VLDL, a finding that would, in part, explain the relevance of hypertriglyceridemia among those homozygous for apoE2.

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