Markedly increased secretion of VLDL triglycerides induced by gene transfer of apolipoprotein E isoforms in apoE-deficient mice

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Abstract **Apolipoprotein E (apoE) plays a key role in the receptor-mediated uptake of lipoproteins by the liver and therefore in regulating plasma levels of lipoproteins. ApoE may also facilitate hepatic secretion of very low density lipoprotein (VLDL) triglyceride (TG). We directly tested the hypothesis that reconstitution of hepatic apoE expression in adult apoE-deficient mice by gene transfer would acutely enhance VLDL-TG production and directly compared the three major human apoE isoforms using this approach. Second generation recombinant adenoviruses encoding the three major isoforms of human apoE (E2, E3, and E4) or a control virus were injected intravenously into apoE-deficient mice, resulting in acute expression of the apoE isoforms in the liver. Despite the expected decreases in total and VLDL cholesterol levels, apoE expression was associated with increased total and VLDL triglyceride levels (E2 > E4 > E3). The increase in TG levels significantly correlated with plasma apoE concentrations. In order to determine whether acute apoE expression influenced the rate of VLDL-TG production, additional experiments were performed. Three days after injection of adenoviruses, Triton WR1339 was injected to block lipolysis of TG-rich lipoproteins and VLDL-TG production rates were determined. Mice injected with control adenovirus had a mean VLDL-TG production rate of 74 ± 7 μmol/h/kg. In contrast, VLDL-TG production rates in apoE-expressing mice were 363 ± 162 μmol/h/kg, 286 ± 175 μmol/h/kg, and 300 ± 84 μmol/h/kg for apoE2, apoE3, and apoE4, respectively. The VLDL-TG production rates in apoE-expressing mice were all significantly greater than in control mice but were not significantly different from each other. In summary, acute expression of all three human apoE isoforms in livers of apoE-deficient mice markedly increased VLDL-TG production to a similar degree, consistent with the concept that apoE plays an important role in facilitating hepatic VLDL-TG production in an isoform-independent manner.**—Tsukamoto, K., C. Maugeais, J. M. Glick, and D. J. Rader. **Markedly increased secretion of VLDL triglycerides induced by gene transfer of apolipoprotein E isoforms in apoE-deficient mice.** ***J. Lipid Res.*** **2000.** 41: **253–259.**

Supplementary key words lipoprotein metabolism • triglycerides • apolipoprotein E • gene transfer

Apolipoprotein E (apoE) is a multifunctional protein synthesized by hepatocytes as well as other cells such as macrophages and astrocytes (1). Most plasma apoE is derived from the liver and is associated with lipoproteins. Plasma apoE plays a key role in the receptor-mediated uptake of apoB-containing lipoproteins by the liver and therefore in regulating plasma levels of lipoproteins (1). It mediates the hepatic uptake of apoB-containing lipoproteins through the interaction with lipoprotein receptors such as the LDL receptor and LRP. ApoE exists in three common isoforms in humans (apoE2, apoE3, and apoE4) that differ in their lipoprotein distribution, plasma metabolism, and receptor binding properties (1).

In addition to its well-established role in mediating uptake of lipoproteins, apoE may also play a role in facilitating hepatic secretion of VLDL triglycerides. ApoE deficient mice accumulate large amounts of triglyceride in their livers (2) and have reduced rates of VLDL triglyceride production compared with wild-type mice (3). Mice deficient in murine apoE that chronically overexpress human apoE3 in the liver have elevated VLDL TG levels and a 50% increase in VLDL-TG production (4). Increased apoE expression by stably transfected rat hepatoma cells is associated with increased VLDL-TG secretion in vitro (4).

In the current study, we tested the hypothesis that acute reconstitution of hepatic apoE expression in apoE-deficient mice through gene transfer would mobilize excess stored hepatic lipid and rapidly increase hepatic VLDL-TG production. We also used this experimental design to directly compare the three major isoforms of human apoE in their acute effects on VLDL-TG production in vivo. Overexpression of each of the major human apoE isoforms markedly increased VLDL-TG production in apoE-

Abbreviations: apoE, apolipoprotein E; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; TG, triglycerides; TC, total cholesterol; LRP, LDL-receptor related protein.

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deficient mice, indicating that hepatic apoE expression acutely promotes hepatic VLDL-TG production in an isoform-independent manner.

METHODS

Construction of recombinant adenoviruses

Construction of recombinant second generation adenoviruses encoding the three human apoE isoforms has been described previously (5). In brief, the three cDNAs (apoE2, E3, and E4) were subcloned into the shuttle plasmid vector in appropriate orientation, and the plasmids were linearized with NheI and cotransfected into 293 cells along with adenoviral DNA which was digested with ClaI. Recombinant adenoviruses were screened by PCR, and plaques positive for the apoE cDNA were subjected to a second round of plaque purification. After confirmation of the presence of apoE cDNA and the absence of wild-type adenovirus, they were further expanded in 293 cells and purified by cesium chloride ultra centrifugation. The control adenovirus AdlacZ carries the β-galactosidase cDNA. The purified viruses were stored in 10% glycerol/PBS at −80°C.

Animal studies

Female and male apoE-deficient mice were obtained from Jackson Laboratory and placed on a normal chow. At 12 weeks of age, they were injected intravenously with 1×10^{11} particles of apoE2, apoE3, apoE4, or control lacZ virus (n = 5 per group per sex). In earlier studies, this dose of adenovirus was shown to result in similar levels of hepatic mRNA for all three human apoE isoforms (5). Blood was obtained from the retroorbital plexus after a 4-h fast prior to virus injection and at 3 and 7 days after injection and collected into a tube containing EDTA, NaN_3, gentamicin, phenylmethylsulfonyl fluoride, and benzamidine (the final concentrations were 2 mm, 0.2%, 0.77%, 1 mm, and 1 mm, respectively).

For the VLDL-TG production studies, 12-week-old female apoE-deficient mice were injected with apoE2, apoE3, apoE4, or control lacZ virus at a dose of 1×10^{11} particles. Three days after injection of viruses, mice were placed on fat-free diet (dry cereal) 4 h prior to injection of Triton WR1339 (Sigma) at a dose of 20 mg in a volume of 100 μl. Triton WR1339 coats triglyceride-rich lipoproteins and prevents lipolysis of triglycerides and has been used extensively for quantitation of VLDL-TG secretion rates in animals (6). Blood was drawn from the retroorbital plexus before and 3 h after injection. Plasma triglycerides and total cholesterol levels were determined by enzymatic methods as described above. VLDL-TG production rates were determined using the change in plasma TG levels as previously described (4, 7).

Lipid and Lipoprotein analysis

Pooled plasma samples (120 μL) were subjected to fast protein liquid chromatography (FPLC) gel filtration (Pharmacia LKB Biotechnology, Uppsala, Sweden) on two Superose 6 columns as previously described (5). Each fraction was collected in 500 μL, and cholesterol and triglyceride concentrations were determined using enzymatic assay kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

The plasma total cholesterol and triglyceride levels were measured enzymatically on a Cobas Fara II (Roche Diagnostic Systems Inc., Nutley, NJ) using Sigma reagents (Sigma Chemical Co., St. Louis, MO) in a CDC-standardized lipid laboratory. Human apoE concentrations were determined by immunoturbidometric assay using regents from Wako run on the Cobas Fara II.

RESULTS

Effects of apoE expression on plasma lipid levels

Recombinant second generation adenoviruses encoding the three human apoE isoforms and a control ade-

TABLE 1. Changes in plasma total cholesterol and triglycerides after gene transfer of apoE

	Total Cholesterol				Triglycerides			
Time	AdLacZ	AdapoE2	AdapoE3	AdapoE4	AdLacZ	AdapoE2	AdapoE3	AdapoE4
	mg lipid/dl				*mg lipid/dl*			
Females	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)	(n = 5)
D0	523.0 (39.7)	522.0 (16.2)	525.0 (15.6)	506.0 (33.4)	69.0 (5.6)	68.0 (2.5)	61.0 (4.3)	63.0 (8.9)
D3	662.0 (9.8)	330[a] (17.5)	89[a] (3.7)	108[a] (21.3)	119[a] (4.8)	585[a] (46.7)	189[a] (12.6)	274[a] (58.5)
D7	545.0 (17.5)	291[a] (38.3)	127[a] (9.0)	121[a] (7.8)	89.0 (7.0)	112[a] (6.8)	80[a] (3.5)	89[a] (7.0)
Males	(n = 8)	(n = 8)	(n = 8)	(n = 8)	(n = 8)	(n = 8)	(n = 8)	(n = 8)
D0	631.3 (41.6)	623.1 (45.9)	634.4 (44.5)	618.8 (50.3)	206.3 (24.4)	201.9 (17.1)	181.9 (16.5)	180.6 (23.2)
D3	754.4 (58.7)	291.8 (31.1)	74.4[a] (3.5)	171.9[a] (26.8)	131.9[a] (12.1)	621.3[a] (87.7)	148.8 (10.2)	516.9[a] (103.6)
D7	806.9[a] (45.3)	263.7[a] (31.5)	174.4[a] (13.2)	145.6[a] (12.5)	103.1[a] (10.1)	156.3 (26.1)	86.3[a] (5.8)	90.6[a] (8.0)
All	(n = 13)	(n = 13)	(n = 13)	(n = 13)	(n = 13)	(n = 13)	(n = 13)	(n = 13)
D0	589.6 (32.5)	584.2 (31.5)	592.3 (31.3)	575.4 (36.1)	153.5 (24.3)	150.4 (21.4)	135.4 (19.7)	135.4 (21.8)
D3	718.9 (37.6)	306.5[a] (20.4)	80[a] (3.2)	147.3[a] (19.9)	126.9 (7.7)	607.3[a] (55.4)	164.2 (9.5)	423.5[a] (73.9)
D7	706.2 (46.1)	274.2[a] (23.7)	156.2[a] (10.9)	136.2[a] (8.7)	97.7[a] (6.8)	139.2 (17.0)	83.8[a] (3.8)	90[a] (5.4)

Values are given as mg lipid/dl, mean ± SEM (in parentheses); D0, pre-inection; D3, 3 days post-injection; D7, 7 days post-injection.

[a] Significantly different from D0, $P < 0.05$.

novirus were injected into chow-fed apoE-deficient mice. Plasma cholesterol and triglyceride levels at baseline and 3 and 7 days after injection are provided in **Table 1.** Injection of the control lacZ adenovirus had little effect on plasma cholesterol or triglyceride levels. As expected, expression of apoE2, apoE3, and apoE4 each resulted in a

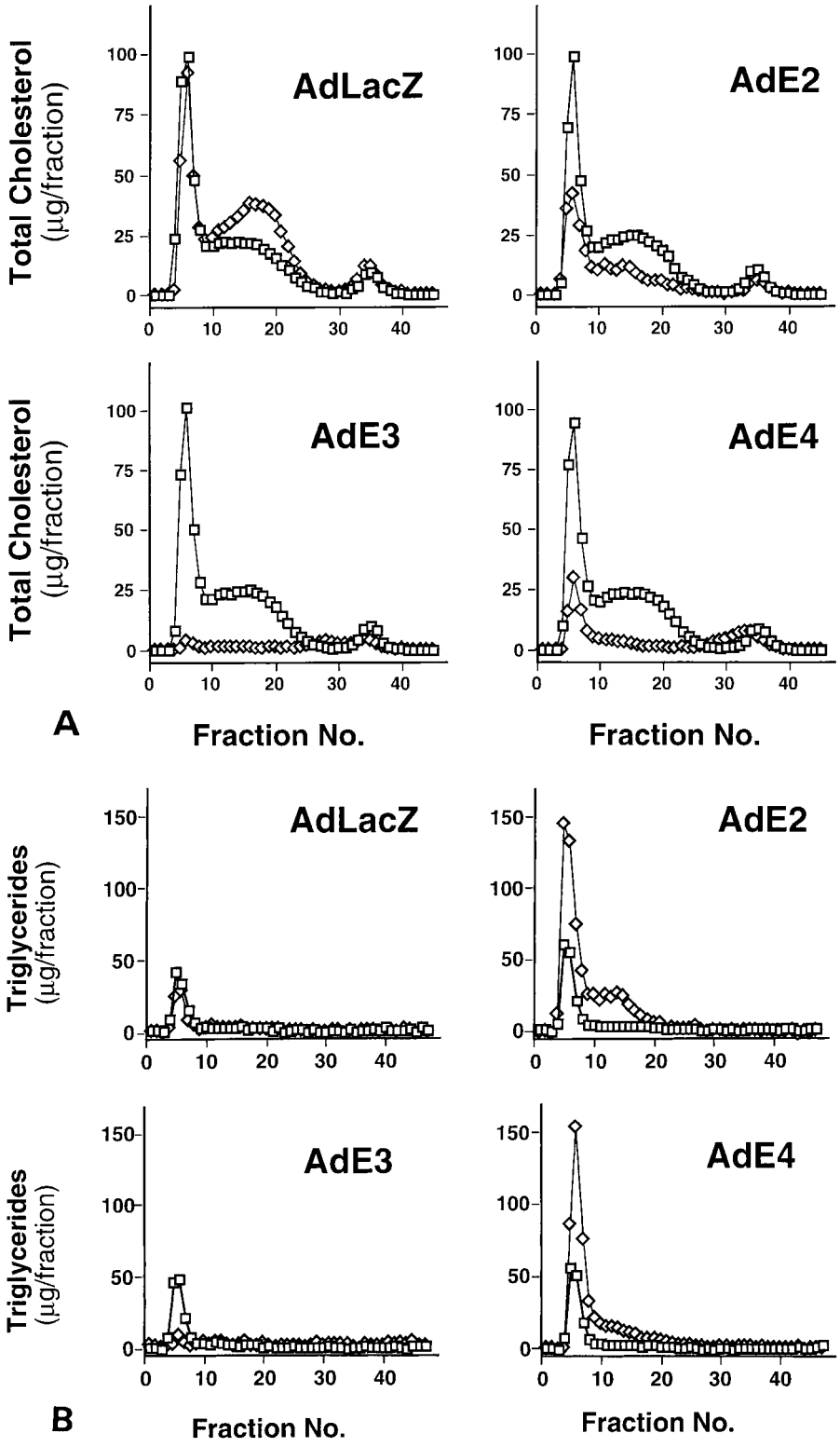

Fig. 1. Distribution of cholesterol and triglycerides in male apoE deficient mice 3 days after injection of AdhapoE2, AdhapoE3, AdhapoE4, and AdlacZ. Pooled plasma samples were subjected to gel filtration using Superose 6 columns and cholesterol (A) and triglycerides (B) in each fraction were measured. Panels AdE2, AdE3, AdE4, and AdLacZ present data from mice injected with apoE2 virus, apoE3 virus, apoE4 virus, and lacZ virus, respectively; squares, before injection of virus; diamonds, 3 days after injection of virus.

marked acute reduction in plasma total cholesterol levels, with the effect of apoE2 somewhat less than that of apoE3 and apoE4. Interestingly, the acute effect of apoE expression on plasma triglyceride levels was considerably different than its effect on plasma cholesterol levels. On day 3 after injection, female mice injected with the human apoE2 adenovirus exhibited greater than an 8-fold increase in plasma triglyceride levels, mice expressing apoE4 had a 4-fold increase in triglycerides, and mice expressing apoE3 had a 3-fold increase in triglycerides, all significantly higher than those seen in mice injected with the control adenovirus. The male apoE-deficient mice had higher levels of triglycerides at baseline prior to injection. Expression of apoE2 and apoE4 in the male mice increased triglyceride levels 3-fold at day 3 and expression of apoE3 had relatively little effect. In order to elucidate which lipoprotein fraction was responsible for the increase of plasma triglycerides in mice injected with the various apoE adenoviruses, plasma obtained prior to and 3 days after adenovirus injection was fractionated by FPLC and cholesterol and triglycerides were quantitated in each fraction. As shown in **Fig. 1A**, cholesterol in VLDL and IDL was substantially reduced by expression of all three apoE isoforms compared to control virus. In contrast, triglycerides were increased in the VLDL fraction by expression of apoE (Fig. 1B). The dissociation of effects on VLDL cholesterol and VLDL triglyceride suggests that apoE expression reduced plasma concentrations of cholesterol-rich VLDL while at the same time increased the plasma levels of TG-rich VLDL.

The correlation between plasma apoE concentration and change in plasma triglyceride levels from baseline is shown in **Fig. 2.** The change in plasma TG level was significantly correlated with the plasma level of apoE2 ($r = 0.81$, $P < 0.001$) with a slope of 7.93. The change in plasma TG level was also significantly correlated with the plasma level of apoE4 ($r = 0.89$, $P < 0.0001$) although the slope of 3.88 was less steep than that seen with apoE2. Finally, there was no correlation of change in plasma TG level with plasma apoE3 ($r = 0.28$, $P = 0.33$). This suggests that the acute effects of hepatic apoE expression on plasma triglyceride levels in apoE-deficient mice are isoform-dependent.

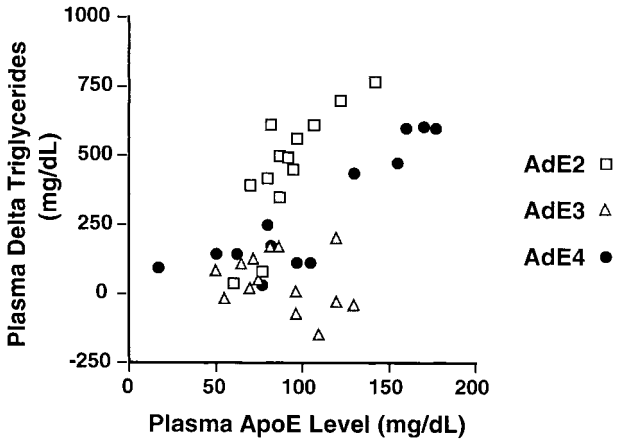

Fig. 2. Correlation between plasma apoE levels and change in plasma triglycerides 3 days after adenovirus injection. The change in triglycerides was calculated by subtracting the plasma triglyceride level before injection of the virus from that 3 days after injection of adenovirus. Delta triglycerides was plotted against the plasma apoE level measured 3 days after injection of virus.

Effects of apoE expression on hepatic VLDL-TG secretion rates

In order to determine whether hepatic apoE expression increased triglycerides by increasing the rate of hepatic VLDL TG secretion, we utilized Triton WR1339 to acutely inhibit the lipolysis of TG-rich lipoproteins. Chow-fed apoE-deficient mice were injected with apoE2, apoE3, apoE4, or control adenoviruses at the same dose as used for the initial experiments. Three days after adenovirus injection, fasting mice were injected with Triton WR1339. Plasma was obtained just before and 3 h after Triton WR1339 injection and analyzed for triglycerides. FPLC analysis demonstrated that the increase in triglycerides after Triton WR1339 was exclusively in the VLDL fraction (**Fig. 3**). The VLDL triglyceride secretion rate in the apoE-deficient mice injected with control virus was 74 ± 7 mg/kg/h, comparable to that previously reported in apoE deficient mice (4). In contrast, expression of apoE2, apoE3, or apoE4 each resulted in markedly increased VLDL-TG secretion rates compared with control virus injected mice (**Fig. 4**). Interestingly, there were no statistically significant differences in VLDL-TG secretion rates among the three apoE isoforms. These data indicate that acute reconstitution of hepatic expression of each of the three human apoE isoforms in apoE-deficient mice acutely increased hepatic VLDL triglyceride secretion to a similar degree.

DISCUSSION

Plasma levels of triglycerides are important determinants of cardiovascular risk (8, 9) and the rate of hepatic VLDL triglyceride production is an important determinant of plasma triglyceride levels. There has been a great deal of investigation in vitro with regard to molecular mechanisms by which hepatic VLDL assembly and apoB-containing lipoprotein secretion occurs (10). However, the factors that regulate the assembly and secretion of VLDL in vivo are not yet completely understood. As reviewed earlier in the introduction, previous studies have suggested that apoE plays a role in the regulation of VLDL-TG secretion. The studies presented here extend previous observations by demonstrating that acute reconstitution of hepatic apoE expression in mice lacking endogenous apoE markedly enhanced hepatic VLDL-TG secretion and suggest that the ability of apoE expression to promote hepatic VLDL-TG secretion may be even greater than previously suspected based on transgenic animals chronically overexpressing apoE.

Investigation of the effects of apoE on hepatic VLDL production in vivo is complex because apoE also has major effects on the plasma metabolism and catabolism of VLDL

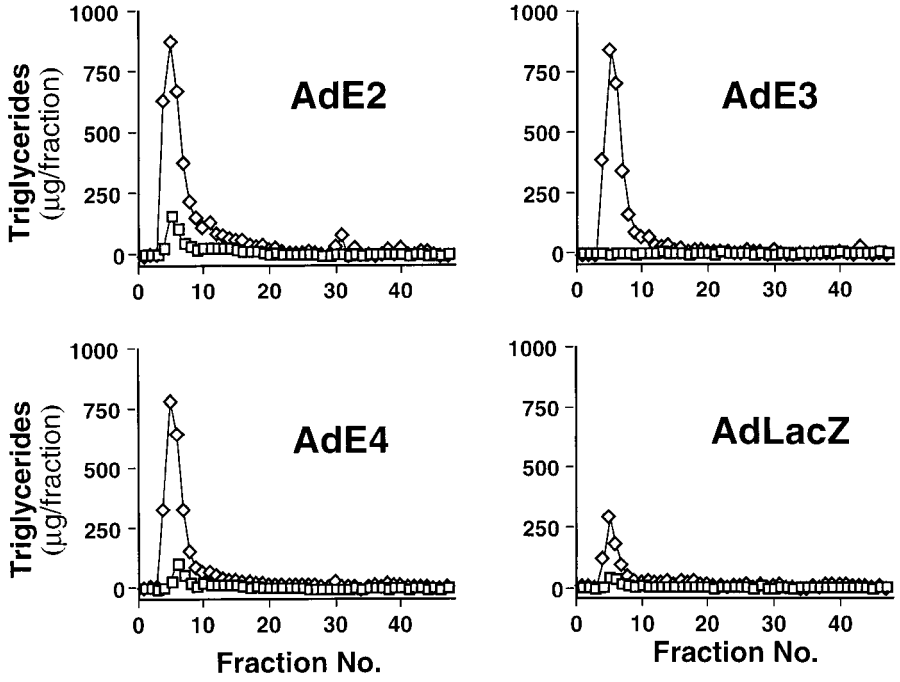

Fig. 3. Lipoprotein distribution of triglycerides after injection of Triton WR1339. Pooled plasma samples were separated by gel filtration using Superose 6 columns and the triglycerides level in each fraction was measured. Panels AdE2, AdE3, AdE4, and AdLacZ present data from mice injected with apoE2 virus, apoE3 virus, apoE4 virus, and lacZ virus, respectively; squares, before injection of Triton WR1339; diamonds, after injection of Triton WR1339.

and its remnants. Therefore, evaluation of lipid levels in animals overexpressing apoE must take into account the potential effects on both lipoprotein catabolism and production. In our studies, expression of apoE dramatically reduced plasma and VLDL/IDL cholesterol levels in apoE-deficient mice, even as it increased plasma and VLDL triglyceride levels. This dissociation of effects on VLDL cholesterol and VLDL triglycerides suggests that apoE had effects on both catabolism (of cholesterol-rich VLDL/IDL) and production (of triglyceride-rich VLDL). The differences among the three apoE isoforms on plasma lipid levels further support this concept. For example, despite the fact that expression of apoE3 markedly increased hepatic VLDL-TG production in apoE-deficient mice, only a small increase was seen in the total plasma TG levels 3 days after vector administration. This is likely due to the fact that apoE3 also dramatically increased the clearance of TG-rich lipoproteins, offsetting the increased VLDL-TG production and resulting in a small net change in plasma TG levels. In contrast, expression of apoE2, which also markedly increased hepatic VLDL-TG production, resulted in a highly significant increase in plasma triglyceride levels 3 days after vector administration, probably due to the fact that clearance of TG-rich VLDL was relatively impaired compared with apoE3. Expression of apoE4 resulted in a significantly greater increase in plasma triglycerides on day 3 compared with apoE3, consistent with the findings that humans with at least one apoE4 allele have increased plasma VLDL remnants (11) and delayed clearance of remnant lipoproteins (12), and that mice expressing apoE4 have reduced VLDL catabolism compared with mice expressing apoE3 (13).

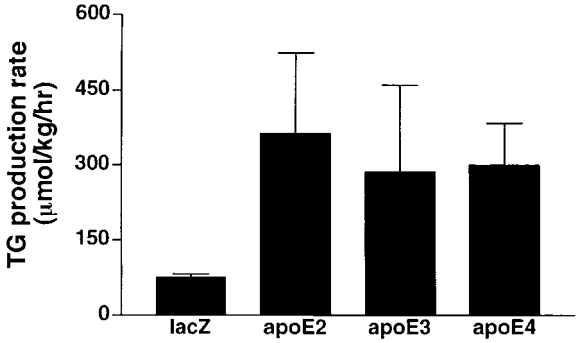

Fig. 4. VLDL triglyceride production rates. The difference in plasma triglyceride levels in plasma obtained prior to Triton WR1339 injection compared with plasma obtained 3 h later was used to calculate the TG production rates. Values ± SEM are shown for each group (n = 3 per group).

An important conclusion of this study is that in contrast to the effect on plasma TG concentrations, the increase in VLDL-TG secretion rate was independent of the isoform of apoE expressed, indicating that all three major human apoE isoforms promoted hepatic VLDL-TG production in vivo to a similar degree. This is consistent with the in vitro observation that all three human apoE isoforms increased VLDL-TG secretion when expressed by transfection in McA-RH7777 rat hepatoma cells (4). Transgenic mice expressing human apoE3 were found to have a 50% increase

in VLDL-TG production, but comparative studies have not been performed in transgenic mice expressing the other forms of human apoE. It is interesting to note that when human apoE2 was overexpressed in wild-type and human apoB transgenic mice, effects on plasma lipoproteins depended on the magnitude of the apoE2 overexpression (14). When these mice were bred to apoE-deficient mice or LDL receptor-deficient mice, the hyperlipidemia was exacerbated (15). Similar results were found by other groups overexpressing human apoE2 on an apoE-deficient background (16, 17). Mice expressing apoE-Leiden on an apoE deficient background are less hypertriglyceridemic than those expressing human apoE2 on the same background (16), suggesting the possibility of differences in VLDL-TG production rates. However, VLDL-TG production rates have not been reported in any of these transgenic mouse models expressing forms of apoE other than wild-type human apoE3. Our data indicate that acute reconstitution of hepatic expression of each of the three human apoE isoforms in apoE-deficient mice acutely increased hepatic VLDL triglyceride secretion to a similar degree. Therefore, the marked differences in plasma TG levels among the three isoforms 3 days after adenovirus injection are not due to differences in VLDL-TG production rates but are likely due to their known differences in catabolism (18, 19). It should be noted, however, that levels of hepatic apoE expression were not directly measured in this study, and because of isoform differences in plasma catabolism, the plasma apoE levels do not reflect expression. Nevertheless, in earlier studies, the same dose of adenovirus was shown to result in similar levels of hepatic mRNA for all three human apoE isoforms (5), and therefore the levels of expression of the three isoforms were probably comparable in the current study. Therefore, the effect of hepatic apoE expression on VLDL-TG secretion is isoform-independent, whereas the effect on clearance of TG-rich lipoproteins is isoform-dependent. Finally, apoE overexpression has been shown to be associated with inhibition of lipoprotein lipase (4, 16) and therefore we cannot exclude the possibility that some of the increase in plasma TG levels in this experiment could have been due to LPL inhibition.

The mechanism(s) by which apoE promotes increased VLDL-TG secretion are unknown. ApoE has been found intracellularly in association with nascent TG-rich lipoproteins (20, 21) and expression of human apoE in insect larvae mediates the formation of more buoyant lipoproteins (22). Therefore, apoE may play an intracellular role in the progressive lipidation of the nascent VLDL particle prior to secretion. Hepatic apoE is apparently recycled by hepatocytes from the extracellular compartment back into the secretory pool (21) through a process that requires binding to heparan-sulfate proteoglycans (HSPGs) (23). This suggests that extracellular apoE could potentially influence VLDL-TG production. Given marked differences among the three major apoE isoforms in binding affinity to HSPGs, our finding that the three apoE isoforms did not differ in their effect on VLDL-TG production suggests that recycling of apoE is unlikely to be required for this effect.

Transgenic mice chronically expressing high-level human apoE3 on the background of absent endogenous murine apoE had VLDL-TG production rates that were 50% greater than those of wild-type mice (6) but were much lower than those of mice acutely expressing apoE3 in our studies. The livers of apoE-deficient mice are known to be enriched in triglycerides (3). Therefore, acute expression of apoE may have resulted in mobilization of excess stored hepatic TG for rapid assembly into nascent VLDL particles. This could explain the differences in absolute VLDL-TG production rates between the acute expression studies presented here and those of mice in which apoE3 is overexpressed chronically (24). These combined results suggest that the magnitude of the effect of apoE expression on hepatic VLDL-TG production might be dependent on the availability of hepatic TG, a plausible hypothesis that is experimentally testable. Interestingly, factors that increase hepatic VLDL secretion also tend to increase hepatic apoE production. Sucrose-rich diets promoted hepatic apoE gene transcription and hepatic VLDL triglyceride production (25). High cholesterol and high saturated fat diets increased hepatic VLDL cholesterol levels and apoE production in rats and mice (26). In light of our and others' data, it is interesting to speculate that hepatic expression of apoE is up-regulated in parallel with increased TG synthesis in order to facilitate the loading of increased TGs onto the assembling VLDL particles. In one study in humans, variation in plasma apoE concentrations accounted for up to 40% of the variation in plasma triglyceride levels (27), consistent with this concept.

In summary, gene transfer and acute hepatic expression of apoE2, apoE3, and apoE4 each resulted in markedly increased hepatic production of VLDL-TG in apoE-deficient mice in an isoform-independent manner. This finding extends previous observations and is consistent with the concept that hepatic apoE production plays an important role in the hepatic assembly and secretion of VLDL and that this effect is independent of apoE isoform.

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