JCI The Journal of Clinical Investigation

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J Clin Invest. 1997;99(11):2672-2681. https://doi.org/10.1172/JCI119456.

Research Article

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Chylomicronemia due to Apolipoprotein CIII Overexpression in Apolipoprotein E-null Mice

Apolipoprotein CIII–induced Hypertriglyceridemia Is Not Mediated by Effects on Apolipoprotein E

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Abstract

The mechanism of apolipoprotein (apo) CIII–induced hypertriglyceridemia remains uncertain. We crossed apoCIII transgenic and apoE gene knockout ($apoE_0$) mice, and observed severe hypertriglyceridemia with plasma triglyceride levels of 4,521±6,394 mg/dl vs. 423±106 mg/dl in $apoE_0$ mice, $P < 0.00001$ for log(triglycerides [TG]). Cholesterols were 1,181±487 mg/dl vs. 658±151 mg/dl, $P < 0.0001$. Lipoprotein fractionation showed a marked increase in triglyceride-enriched chylomicrons+VLDL. This increase was limited to the lowest density (chylomicrons and S_f 100–400) subfractions. Intermediate density lipoproteins (IDL)+LDL increased moderately, and HDL decreased. There was no significant increase in triglyceride production in apoCIII transgenic/$apoE_0$ mice. The clearance of VLDL triglycerides, however, was significantly decreased. Lipoprotein lipase in postheparin plasma was elevated, but activation studies suggested LPL inhibition by both apoCIII transgenic and apoCIII transgenic/$apoE_0$ plasma. ApoCIII overexpression also produced a marked decrease in VLDL glycosaminoglycan binding which was independent of apoE. The predominant mechanism of apoCIII-induced hypertriglyceridemia appears to be decreased lipolysis at the cell surface. The altered lipoprotein profile that was produced also allowed us to address the question of the direct atherogenicity of chylomicrons and large VLDL. Quantitative arteriosclerosis studies showed identical results in both apoCIII transgenic/$apoE_0$ and $apoE_0$ mice, supporting the view that very large triglyceride-enriched particles are not directly atherogenic. (*J. Clin. Invest.* 1997. 99:2672–2681.)

Key words: apolipoprotein E • apolipoprotein C • atherosclerosis • lipoproteins, VLDL • mice, transgenic

Introduction

An increasing body of evidence links overexpression of apolipoprotein (apo) CIII with hypertriglyceridemia. Soon after its identification as a distinct protein (1, 2), and in the absence of apoE, apoCIII was shown to inhibit the activation of lipoprotein lipase (LPL)[1] by apoCII (3, 4). ApoCIII was subsequently shown to inhibit hepatic lipase as well (5). Elevation of VLDL apoCIII, or of the apoCIII/apoCII ratio, has been observed in clinical hypertriglyceridemia (6–8). Patients with hereditary deficiency of apoCIII (and apoAI) had low plasma triglycerides (TG) and accelerated clearance of both postprandial triglyceride-rich lipoproteins (TGRL) and VLDL with loss of the normal inhibitory effect of serum on lipoprotein lipase (LPL) activity (9, 10). Normotriglyceridemic relatives of hypertriglyceridemic probands had decreased VLDL catabolism (11), and genetic linkage studies showed an association of DNA polymorphisms present at the apoCIII gene locus with hypertriglyceridemia (12, 13). One such polymorphism has been shown to abolish the normal inhibition of apoCIII gene expression produced by insulin (14).

In experimental liver perfusions, apoCIII was shown to inhibit hepatic uptake of triglyceride-rich emulsions and lipoproteins, both by displacing apoE, and via an apparent apoE-independent mechanism (15, 16). Each of the apoCs has been shown to decrease binding of beta-migrating very low density lipoproteins (β-VLDL), a model of postlipolysis lipoprotein remnants, to a remnant receptor, the LDL receptor–related protein (LRP) (17, 18). Each of the apoCs has also been shown to decrease the apoE-mediated binding of human VLDL and intermediate density lipoprotein (IDL) to the LDL receptor (19, 20). Despite this work, the physiological effects of apoCIII remain unclear. Whether decreased lipolysis or decreased receptor-mediated lipoprotein clearance predominates in vivo remains the subject of controversy.

A series of investigations in transgenic mice has confirmed the potential importance of apoCIII, while leaving open questions regarding its mechanism of action. Transgenic mice expressing human apoCIII at less than half the normal human level had a doubling of plasma TG, an effect well beyond any postulated biochemical effect on LPL (21). Moreover, while VLDL triglyceride clearance in vivo was markedly delayed, VLDL lipolysis in vitro appeared to be normal, leading to the supposition that decreased particulate uptake, related to the displacement of apoE, was the predominant mechanism (22, 23). This supposition, however, appeared to be contradicted by the absence of dramatic hypertriglyceridemia in apoE gene knockout ($apoE_0$) mice (24, 25). Furthermore, apoCIII gene knockout mice showed very rapid catabolism of triglyceride-

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Received for publication 30 December 1996 and accepted in revised form 18 March 1997.

J. Clin. Invest.

0021-9738/97/06/2672/10 $2.00
Volume 99, Number 11, June 1997, 2672–2681

1. *Abbreviations used in this paper:* $apoE_0$, apolipoprotein E gene knockout; $CIII/E_0$, human apoCIII transgenic/$apoE_0$; HuCIII Tg, human apoCIII transgenic; IDL, intermediate density lipoproteins; LPL, lipoprotein lipase; S_f, Svedberg units of flotation; TG, triglycerides; TGRL, triglyceride-rich lipoproteins; WT, wild-type.

rich lipoproteins, consistent with increased lipolysis (26). On the basis of in vitro binding studies, we proposed decreased association of VLDL with cell-surface glycosaminoglycans, and consequent decreased lipolysis in vivo, as the predominant mechanism of hypertriglyceridemia in both human apoCII transgenic and human apoCIII transgenic (HuCIII Tg) mice (27, 28). ApoCI transgenic mice exhibit a different phenotype with accumulation of cholesterol-enriched VLDL, a phenotype that is consistent with a remnant clearance defect. Interestingly, VLDL from these mice showed normal glycosaminoglycan binding in the in vitro assay (28).

To address the issue of the independent effects of apoCIII on triglyceride metabolism, we have crossed HuCIII Tg and $apoE_0$ mice, and have conducted a detailed analysis of the lipoprotein phenotype. We have also performed a quantitative arteriosclerosis assay to assess the consequences of the altered lipoprotein particle composition and size distribution present in these animals.

Methods

Transgenic and gene knockout mice. High-expressor HuCIII Tg line 3707, in a mixed C57BL6 × CBA background, and $apoE_0$ mice (129OLA mated into the same background and then propagated by brother-sister mating) were the generous gift of Jan L. Breslow (Rockefeller University, New York) (22, 25). The animals were mated, and the presence of the human apoCIII transgene was determined by PCR of tail-derived DNA. Double heterozygotes were backcrossed to $apoE_0$ to produce HuCIII Tg animals homozygous for $apoE_0$. All animals (both human apoCIII transgenic/$apoE_0$ [$CIII/E_0$] and $apoE_0$) used in the subsequent investigations were the progeny of two $CIII/E_0$ males mated to $apoE_0$ females. $CIII/E_0$ and $apoE_0$ animals were produced in approximately equal numbers. All HuCIII Tg mice used were of mixed C57BL/6 × CBA background.

Animal protocols. Animals were caged in an approved animal care facility with a 7 a.m.–7 p.m. period of light. Animals were fed a standard mouse chow diet containing 4.5% fat (9% of calories) and 0.02% cholesterol (Ralston Purina Co., St. Louis, MO). Access to food was ad libitum, except for fasting blood specimens. Both male and female mice were used in approximately equal ratios in all experiments, except where specified. Animals were anesthetized with methoxyflurane for retroorbital phlebotomy and intravenous femoral injections. 100 μl of plasma was used for analyses, and animals were allowed to recover for at least 10 d between phlebotomies.

Phlebotomy times were based on the nocturnal feeding behavior of mice. The fed state was defined as 9:00 a.m. and the fasted state as 5:00 p.m., after the removal of food at 8:00 a.m. Fed state bloods on individual animals were obtained when they were 3 mo old. All animals were age- and sex-matched in all experiments (see below for ages).

Lipoprotein analysis. Triglyceride and cholesterol concentrations were performed using commercial kits (Boehringer Mannheim Biochemicals, Indianapolis, IN) on an autoanalyzer (model 705; Hitachi Ltd., Tokyo, Japan). Phospholipids were determined using a commercial kit (Phospholipids B; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Chylomicrons+VLDL ($d < 1.006$ g/ml), IDL+LDL ($d = 1.006$–1.063 g/ml), and HDL ($d = 1.063$–1.21 g/ml) were separated by sequential density ultracentrifugation of pooled mouse plasma obtained at 16 wk of age (29). Protein concentrations in lipoprotein fractions were determined (BCA protein assay; Pierce Chemical Co., Rockford, IL). Gel filtration chromatography was performed on 500 μl of pooled mouse plasma, obtained in the fasted state from mice that were 6–7 mo old, using two columns in series (Superose 6; FPLC, Pharmacia LKB Biotechnology, Piscataway, NJ). 0.5-ml fractions were collected, and cholesterol and triglyceride levels were determined as described above. Each pool contained plasma from five mice. Plasma samples from $CIII/E_0$ mice were assigned to one of two pools, based on the presence or absence of overt lipemia.

Subfractionation of TGRL. Chylomicrons were defined as density < 1.006 lipoproteins isolated from a 20-min repeat ultracentrifugation of the chylomicrons+VLDL fraction. 1 ml of lipemic top was removed from a total vol of 6 ml; the procedure was otherwise as described (29). The remaining VLDL were subfractionated by nonequilibrium density gradient ultracentrifugation as described (30, 31).

Statistics. All comparisons were by *t* test. Two-tailed *P* values are reported. Because of the marked skewing of the triglyceride data, *P* values for the natural logarithm of the triglyceride values are reported when indicated.

Apolipoprotein analysis. ApoB100, apoB48, and apoAI and apoCII/apoCIII were quantitated via SDS-PAGE of variable amounts of nondelipidated lipoproteins from the above three $CIII/E_0$ and three $apoE_0$ pools of VLDL using precast 4–20% polyacrylamide gradient gels with a 3% stacking gel (Bio-Rad Laboratories, Inc., Hercules, CA). A constant amount of protein was loaded for each lipoprotein species on each gel. Gels were run with 5, 10, and 20 μg of VLDL loaded. The ratio of scan density between different apolipoprotein species was maintained on gels with different loads. LDL gels were run with 5 μg of protein only. Gels were fixed overnight in 50% methanol, 10% acetic acid, and were stained using Coomassie blue as follows: staining for 4 h in 50% methanol, 10% acetic acid, 0.05% Coomassie brilliant blue 250, and destaining for 4 h in 5% methanol and 7% acetic acid followed by water (overnight). The gels were scanned using a laser densitometer and Image QuaNT software (Molecular Dynamics, Sunnyvale, CA). The local median of background, based on a box drawn around the band, was subtracted from the scanned densities. No adjustment was made for differential chromogenicity of the proteins, limiting conclusions to relative changes in apolipoprotein composition. Isoelectric focusing was performed, as described, on ultracentrifugally isolated VLDL from wild-type (WT) HuCIII Tg, $apoE_0$, $CIII/E_0$-nonlipemic, and $CIII/E_0$-lipemic mice (28, 32). Each of the samples was run on a separate tube gel, and therefore differed slightly in the focusing position of the apolipoproteins.

Triglyceride production. Triglyceride production studies were performed on four 6-mo-old mice of each genotype, according to a technique described in HuCIII Tg mice (22). Studies were done in the postabsorptive state, between the hours of 11 a.m. and 2 p.m. Triton WR 1339, 500 mg/kg, was injected simultaneously with 250 μCi of [^{3}H]glycerol (NET 848; New England Nuclear, Boston, MA). 60 μl of blood was drawn before injection (for determination of plasma cholesterol and triglycerides) and at 30, 60, and 90 min. 25 μl of plasma from each sample was extracted by the method of Folch (33), and the amount of ^{3}H in triglycerides was determined by thin layer chromatography.

Liver triglyceride content. Quantitation of liver triglycerides was performed using a kit according to the manufacturer's instructions (Triglyceride GPO-Trinder cat. #337; Sigma Chemical Co., St. Louis, MO). A glycerol standard (cat. #G1394; Sigma Chemical Co.) and a triglyceride standard (Precical cat. #620213; Boehringer Mannheim) were used. The entire liver was weighed and then homogenized in a final volume of 10 ml with the addition of physiological saline solution. Absorption due to background and glycerol content has been subtracted from the reported values. Five animals of between 10 and 11 mo of age were studied for each genotype.

Triglyceride clearance. Triglyceride clearance was examined in all animals by the clearance of radiolabeled triglycerides from a single preparation of in vivo–labeled VLDL from $CIII/E_0$ animals, prepared as described (27). Characterization of the tracer by thin layer chromatography (27) revealed that 95.1% of the label was in triglycerides, 2.4% in fatty acids, 1.4% in phospholipids, and 1.1% in cholesteryl esters. $ApoE_0$ VLDL could not be used as a tracer because its triglyceride content was too low to be suitable as a marker of lipolysis. WT or HuCIII Tg VLDL tracers were felt to be poorer models for the tracee because of the presence of apoE. Five animals between 10 and 11 mo of age were studied for each genotype. Results are reported normal-

Table I. Plasma Lipids in CIII/E_0 and ApoE_0 Mice

	CHOL	CHOL range	TG	TG range	*n*
	mg/dl	*mg/dl*	*mg/dl*	*mg/dl*	
CIII/E_0, fast	1181±487	520–2380	4521±6394	360–21280	23
ApoE_0, fast	658±151	380–960	423±106	300–620	13
Overlap, *n*		8		2	
P	0.00005		0.000001*		
CIII/E_0, fed	1018±345	556–2040	2742±4010	364–17800	19
ApoE_0, fed	588±167	188–988	302±199	140–820	27
Overlap, *n*		11		4	
P	0.00004		0.0000001*		

Overlap refers to the number of animals with values within the range of both genotypes. **P* values are reported for the natural logarithm of the triglyceride values. *CHOL*, cholesterol.

ized by the zero time intercept value, which was determined for each animal's data separately by a sum of two exponentials. Normalization to the first measured (2-min) value did not significantly alter the conclusions.

Postheparin lipolytic activity. Five animals each of WT, HuCIII Tg, apoE_0, CIII/E_0 lipemic (chylos+) and CIII/E_0 nonlipemic (chylos−) were studied. Animals were 6–9 mo old. Postheparin plasma was obtained 3 min after the intravenous injection of 10 U of bovine heparin. Chylomicrons were removed by the addition of an equal volume of $d = 1.063$ density solution, and centrifugation for 5 min at 14,000 rpm in an Eppendorf microcentrifuge kept at 4°C. The infranatant 50% was removed with a syringe and tested for LPL and hepatic lipase activities using a gum arabic emulsion essentially as described (34). 20 μl of infranatant postheparin plasma was used for each assay (in duplicate). For the LPL assay, the emulsion was maximally activated with pooled normal mouse plasma (20 μl/sample).

LPL activation. Activation of LPL was assayed for WT, HuCIII Tg, apoE_0, and CIII/E_0 plasmas. Animals were 6–8 mo old. Partially purified mouse LPL was prepared as described (27). Aliquots of mouse LPL were activated with varying amounts of pooled mouse plasma from five mice for each of the above genotypes. 2-, 5-, 10-, 20-, and 40-μl aliquots of each plasma pool were used along with plasma-free controls. Assays were performed in duplicate, which showed close agreement. The assay was then repeated using 5-μl plasma aliquots from five individual mice of each genotype.

VLDL glycosaminoglycan binding. In vivo–labeled VLDL (27) were assessed for binding to heparin-conjugated agarose (heparin-Sepharose CL-6B; Pharmacia LKB Biotechnology), a model for the cell-surface glycosaminoglycan matrix, as described (28). Animals were 6–10 mo old.

Arteriosclerosis. A quantitative arteriosclerosis assay was performed in a blinded manner on 16 female CIII/E_0 and 16 female apoE_0 mice using a minor modification of a described protocol (35). All mice were on chow diets, as described above, and were killed at precisely 16 wk of age. Hearts were perfused first with phosphate-buffered saline, and then with buffered formaldehyde under physiologic pressure (~ 100 mmHg) via cardiac ventricular puncture. The heart and proximal aorta were isolated and fixed for at least 5 d in phosphate-buffered formaldehyde. After fixation, hearts were embedded in 25% gelatin, and were cryotome-sectioned. 12-μm thick sections were cut consecutively from the level of the aortic valve leaflets up to 480 μm above the leaflets in the aortic sinus. Every other section was discarded. Four successive retained sections were mounted on a slide. One section from each slide was chosen for analysis based on contiguity of atheroma. If more than one section was suitable, the first section was used. The areas of five sections (from each of the five slides which were obtained) were then averaged.

Results

Production of human apoCIII transgenic/apoE_0 (CIII/E_0) mice and resultant lipid phenotype. A high-expressor human apoCIII transgene was crossed into the apoE_0 background as described above. To control for the effects of genetic diversity, the CIII/E_0 and apoE_0 mice studied were littermates. Plasma cholesterol and triglyceride levels in both the fed and fasted states are shown in Table I. TG levels showed increased variability, but were, on average, 10-fold elevated in CIII/E_0 vs. apoE_0 animals with 4 out of 23 values >10,000 mg/dl in the fasted state. Cholesterol levels approximately doubled.

Lipoprotein fractionation. Lipoproteins were isolated by ultracentrifugation of three distinct plasma pools from each

Table II. Lipoprotein Profile of CIII/E_0 and ApoE_0 Mice

	Plasma		CHYLOS+VLDL					IDL+LDL				HDL			
	CH	TG	CH	TG	PL	PR	CH/TG	CH	TG	PL	PR	CH	TG	PL	PR
CIII/E_0	1240	12480	589	5724	957	511	1.10	167	56	155	117	8	7	37	24
CIII/E_0	1160	4840	692	4000	631	364	0.17	193	64	143	126	10	11	42	30
CIII/E_0	1100	1520	729	993	430	244	0.73	244	99	173	133	9	12	17	27
Mean	**1167**	**6280**	**670**	**3572**	**673**	**373**	**0.34**	**201**	**73**	**157**	**125**	**9**	**10**	**32**	**27**
SD	70	5620	72	2394	266	134	0.35	39	23	15	8	1	3	13	3.4
Percent			13	67	13	7		36	13	28	23	11	13	41	35
ApoE_0	640	400	414	151	208	127	2.7	132	36	87	74	18	14	16	55
ApoE_0	620	380	450	144	213	112	3.1	109	38	74	66	21	13	33	61
ApoE_0	660	420	422	190	172	119	2.2	107	23	72	60	16	11	15	48
Mean	**640**	**400**	**428**	**162**	**198**	**119**	**2.7**	**116**	**32**	**78**	**66**	**18**	**12**	**21**	**55**
SD	20	20	19	25	22	6	0.46	14	8	8	7	2.6	1.2	9.9	6
Percent			47	18	22	13		40	11	27	22	17	11	20	52
P			0.03	0.03	0.04	0.03	0.002	0.04	0.10	0.004	0.001	0.03	0.26	0.3	0.003

Three distinct plasma pools were characterized for each genotype. *CH*, cholesterol; *TG*, triglycerides; *PL*, phospholipids; *PR*, protein. All values are in mg/dl.

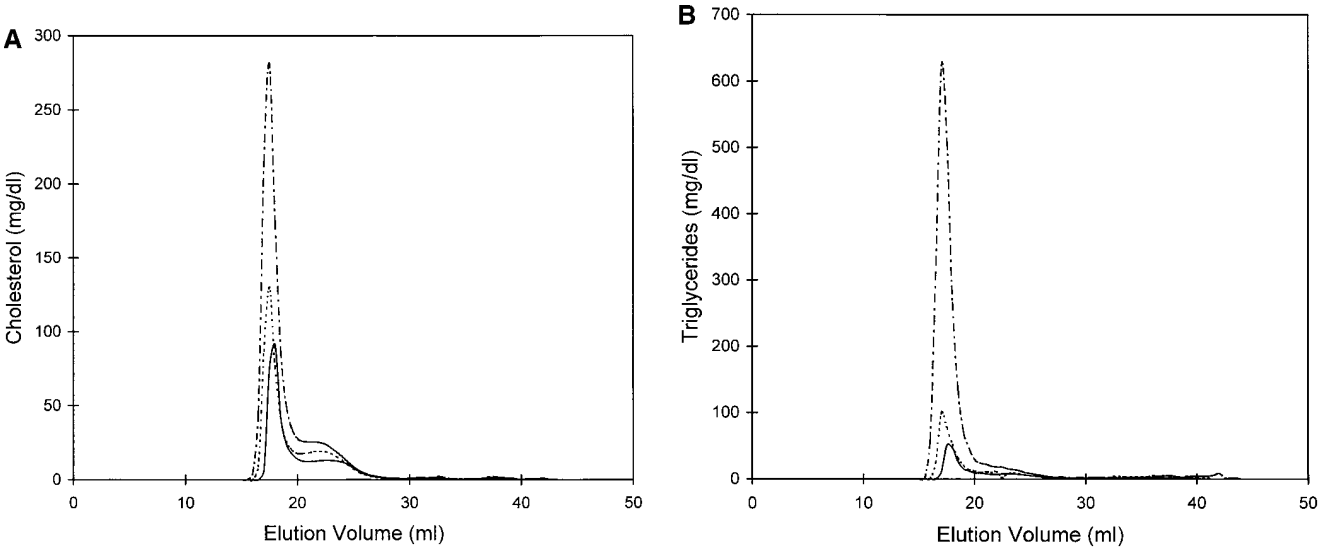

Figure 1. Cholesterol (*A*) and triglyceride (*B*) content of gel filtration chromatography fractions obtained from pooled plasma from lipemic and nonlipemic CIII/E_0 mice and apoE_0 controls. The uninterrupted line shows the apoE_0 profile, the dotted line CIII/E_0 nonlipemic, while the line with alternating dots and dashes reflects the lipemic CIII/E_0 pool. Fractions 15–20 represent chylomicrons+VLDL, fractions 20–25 correspond to LDL, and fractions 30–35 are equivalent to HDL. Chylomicrons are increased in CIII/E_0 plasma, and show an increased TG/CHOL ratio, particularly in the lipemic pool. Chylos+VLDL begin to elute earlier, consistent with the presence of larger particles. LDL are moderately increased in CIII/E_0, while HDL are low in all pools.

genotype. Results appear in Table II. As expected, chylomicron+VLDL triglycerides, on both an absolute and percentage basis, were strikingly elevated in CIII/E_0 mice. Because of the decreased recovery of triglycerides in the most lipemic CIII/E_0 plasma pool, this is certainly an underestimate. The absolute amount of cholesterol increased, but the relative amount declined. The VLDL cholesterol/TG ratio was about eightfold reduced ($P = 0.002$). The ratio of phospholipid plus protein to cholesterol plus triglycerides also decreased (0.28 ± 0.098 in CIII/E_0 vs. 0.54 ± 0.057 in apoE_0, $P < 0.02$), consistent with the presence of larger particles. IDL+LDL cholesterol was high in apoE_0 mice, and increased further, by $\sim 70\%$, in the CIII/E_0 animals. IDL+LDL particle composition, on a relative basis, was essentially unchanged. HDL cholesterol was low in the apoE_0 animals, as has been described (25). In the CIII/E_0, HDL-cholesterol and protein fell further (by about half).

Gel filtration chromatography was performed on pooled plasma from overtly lipemic CIII/E_0, nonovertly lipemic CIII/E_0, and apoE_0 mice. Results are shown in Fig. 1, *A* for cholesterol and *B* for triglycerides. Cholesterol was markedly increased in the lipemic CIII/E_0 pool, and moderately increased in the nonlipemic pool. Triglycerides in chylomicrons+VLDL were dramatically increased in the lipemic pool, out of proportion to cholesterol. In both CIII/E_0 pools TGRL began to elute earlier, consistent with the presence of larger particles. LDL

Table III. Subfractionation of Density < 1.006 g/ml Lipoproteins

	CH	TG	PL	PR	(PR+PL)/CH+TG)
Chylos, CIII/E_0	356±49	2371±1692	336±187	142±49	0.13±0.02
Chylos, ApoE_0	152±25	102±19	65±14	34±11	0.26±0.02
P	0.003	0.15	0.13	0.06	0.0036
S_f 100–400, CIII/E_0	42±29	151±19	53±16	32±5	0.28±0.08
S_f 100–400, Apo/E_0	15±2.6	11±4	14±2	9.1±0.4	0.53±0.05
P	0.25	0.006	0.05	0.02	0.011
S_f 60–100, CIII/E_0	58±46	73±32	45±15	39±11	0.38±0.08
S_f 60–100, Apo/E_0	52±19	19±6	25±5	16±3.2	0.37±0.05
P	0.84	0.1	0.12	0.08	0.84
S_f 20–60, CIII/E_0	73±55	42±22	62±26	65±13	0.62±0.16
S_f 20–60, Apo/E_0	90±16	26±5.4	49±6.5	37±6.1	0.42±0.01
P	0.66	0.34	0.48	0.04	0.10

Subfraction composition (in mg/dl) of the CHYLOS+VLDL fractions from Table II. The ratio of protein and phospholipid, which are predominantly on the lipoprotein particle surface, to cholesterol (mostly present as cholesteryl esters) and triglycerides, which are mostly in the core, is inversely correlated with particle size. *Chylos*, chylomicrons.

Table IV. Chylos+VLDL Apolipoproteins (Arbitrary Density Units)

	ApoB48	ApoAI	ApoC
$CIII/E_0$	2950±1113	4122±958	31349±6474
$ApoE_0$	4905±390	15186±2217	23337±5652
P	0.10	0.004	0.18

A constant amount of protein was loaded. The lower levels of core (apoB48) protein in the III/E_0 samples are consistent with the presence of larger particles. There also appears to be a direct displacement of apoAI by apoC. *CHYLOS*, chylomicrons.

were increased in the $CIII/E_0$ pools, more so in the lipemic pool. HDL were very low in $apoE_0$ plasma as well as both $CIII/E_0$ pools.

Subfractionation of TGRL. Density < 1.006 lipoproteins from the same pools were further subfractionated by nonequilibrium density gradient ultracentrifugation. Results appear in Table III. The marked increase in triglycerides in the $CIII/E_0$ animals appeared to be accounted for primarily by an increase in the chylomicron and Svedberg units of flotation (S_f) 100–400 density ranges. Based on the ratio of phospholipid and protein to cholesterol and triglycerides, lipoprotein particles within the chylomicron and S_f 100–400 density ranges were significantly larger in the $CIII/E_0$ mice. The ratios in the S_f 60–100 subfraction were similar, while in the S_f 20–60 density range, particles appeared to be smaller in the $CIII/E_0$ animals.

Apolipoprotein composition. Apolipoproteins were quantitated by SDS-PAGE of chylomicrons+VLDL and IDL+LDL lipoprotein density fractions. Scan data for chylomicrons+VLDL are presented in Table IV in arbitrary density units. ApoB was evident only as apoB48 in chylomicrons+VLDL from both genotypes. Some apoAI was present in the $apoE_0$ VLDL, as has been described (25); this presence was markedly reduced in the $CIII/E_0$ chylomicrons+VLDL. ApoB tended to be lower, and apoCs tended to be higher in the $CIII/E_0$ chylomicrons+VLDL, consistent with the presence of larger particles, though these changes did not reach statistical significance. The findings were similar, but more extreme, in IDL+LDL (Fig. 2) with no apoAI visible in the $CIII/E_0$ IDL+LDL, and very little apoC visible in the $apoE_0$ IDL+LDL. ApoB, as a fraction of total IDL+LDL protein, was unchanged. Isoelectric focusing of VLDL (Fig. 3) confirmed these findings with increased apoAI in $apoE_0$ VLDL, which decreased in nonlipemic $CIII/E_0$ VLDL and disappeared in lipemic $CIII/E_0$ VLDL. ApoCII in HuCIII Tg VLDL was decreased, as has been described (22, 28), but appeared essentially unchanged in $apoE_0$ and $CIII/E_0$ VLDL, arguing against selective displacement of apoCII as the cause of the observed hypertriglyceridemia. The relative amounts of mouse and human apoCIII appear to be the same in the $CIII/E_0$ and HuCIII Tg VLDL. An extra band above $CIII_0$ was evident in HuCIII Tg VLDL. This band has also been observed by others, and is of unknown identity (22).

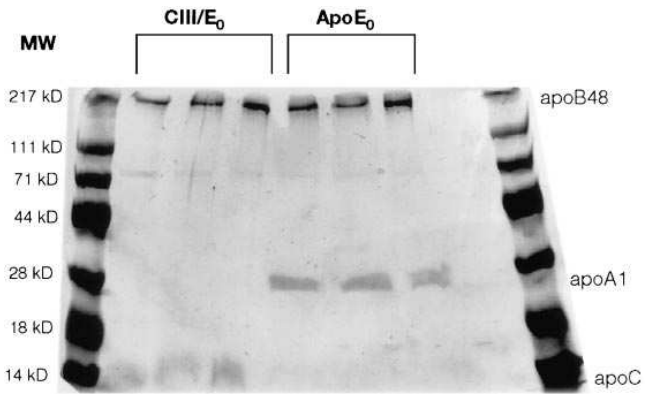

Figure 2. SDS-PAGE of 5 μg (protein) of IDL+LDL from three pools of plasma (each) from $CIII/E_0$ and $apoE_0$ mice. Increased ApoAI is noted in the $apoE_0$ pools and increased apoCs in the $CIII/E_0$ pools. ApoB quantity was the same, and consisted of apoB48 only in IDL+LDL from both genotypes.

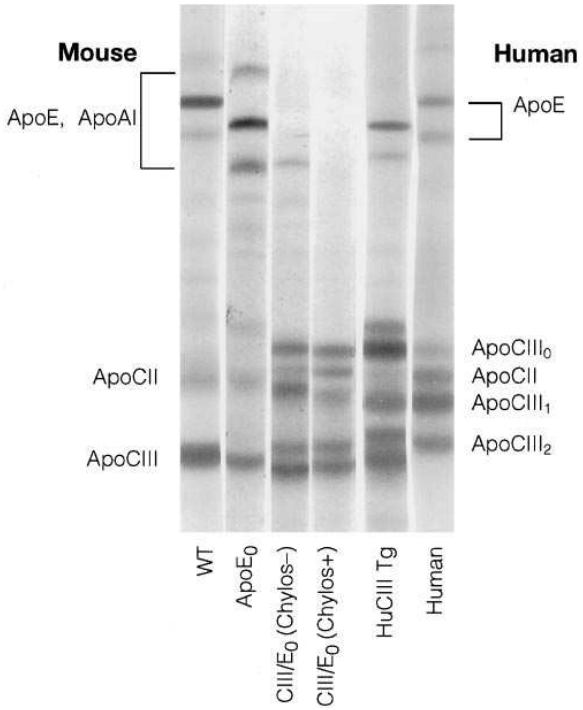

Figure 3. Isoelectric focusing of a constant amount of delipidated VLDL apoproteins from WT, $apoE_0$, $CIII/E_0$ nonlipemic (*Chylos*−), $CIII/E_0$ lipemic (*Chylos*+), and HuCIII Tg mice as well as a normal human.

Triglyceride production. To distinguish between increased production and decreased clearance of triglycerides as the cause of hypertriglyceridemia, triglyceride production was measured as the incorporation of radiolabeled glycerol into plasma triglycerides. The clearance of plasma triglycerides was pharmacologically inhibited with Triton (Table V). Despite the > 10-fold difference in total plasma triglycerides, there was no significant difference in radiolabeled triglycerides at any time point, indicating that increased triglyceride production does not prominently contribute to the observed phenotype. The incorporation of radiolabeled glycerol into plasma triglycerides did not appear to be artifactually depressed because of increased hepatic triglyceride content in $CIII/E_0$ mice. $CIII/E_0$ mice had 44±32 mg of triglycerides per g of wet liver wt vs. 62±42 mg/g for $apoE_0$ animals ($n = 5$ for each, $P = 0.42$).

Table V. Plasma Triglyceride Production (in cpm of ^{3}H-TG)

	30 min	60 min	90 min	CH	TG	BW
CIII/E_0	1246	7139	8882	977	4335	35
SD	585	1532	4059	316	2606	7
ApoE_0	956	4748	6180	610	320	29
SD	625	2490	3642	80	41	2.7
P	0.52	0.15	0.36	0.11	0.05	0.19

BW, body weight. *CH* and *TG*, plasma cholesterol and triglyceride levels in mg/dl immediately before injection of the tracer.

Triglyceride clearance. Triglyceride clearance was estimated by the injection of ^{3}H-TG radiolabeled VLDL produced in CIII/E_0 animals (Fig. 4). A statistically significant increase in plasma cpm was evident at 40 and 75 min after infusion of the tracer in the CIII/E_0 mice, with a trend at other points indicating decreased VLDL triglyceride clearance. The mechanism of decreased triglyceride clearance was assessed by a variety of measures.

Plasma lipase activities. Postheparin plasma from WT, HuCIII Tg, apoE_0, CIII/E_0 nonlipemic, and CIII/E_0 lipemic mice was isolated and purified free of chylomicrons by centrifugation. Plasma postheparin hepatic lipase and LPL activities were determined and revealed no decrease in lipase activity in HuCIII Tg, apoE_0, and either lipemic or nonlipemic CIII/E_0 mice (Fig. 5). Indeed, there was a large and statistically significant increase in LPL activity in the HuCIII Tg mice versus the WT. Lipase activities in the CIII/E_0 animals were no lower than in apoE_0 animals. Interestingly, activities in the lipemic and nonlipemic CIII/E_0 mice were identical.

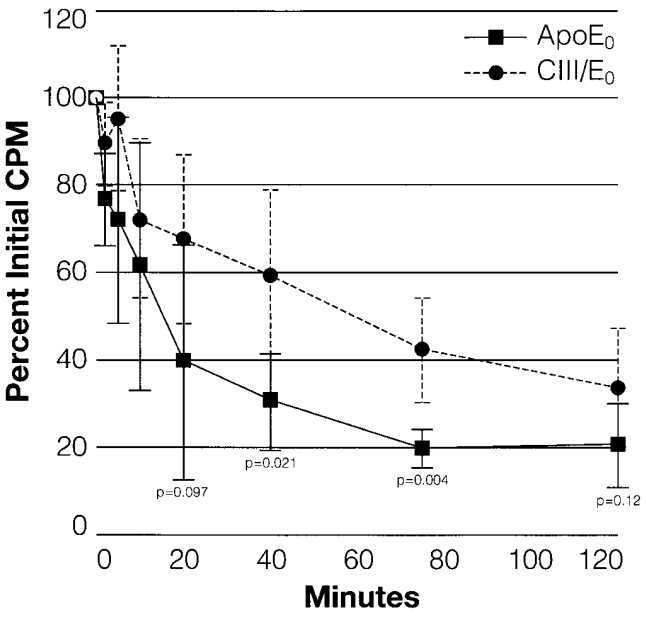

Figure 4. Clearance of ^{3}H-TG in CIII/E_0 VLDL from the plasma of apoE_0 and CIII/E_0 mice. The solid square with the uninterrupted line indicates apoE_0, while the solid circle with the dotted line indicates CIII/E_0. Means±SD of percent initial CPM are displayed for each time point after normalization to a calculated zero time point, determined by fitting each animal's data separately by a sum of two exponentials.

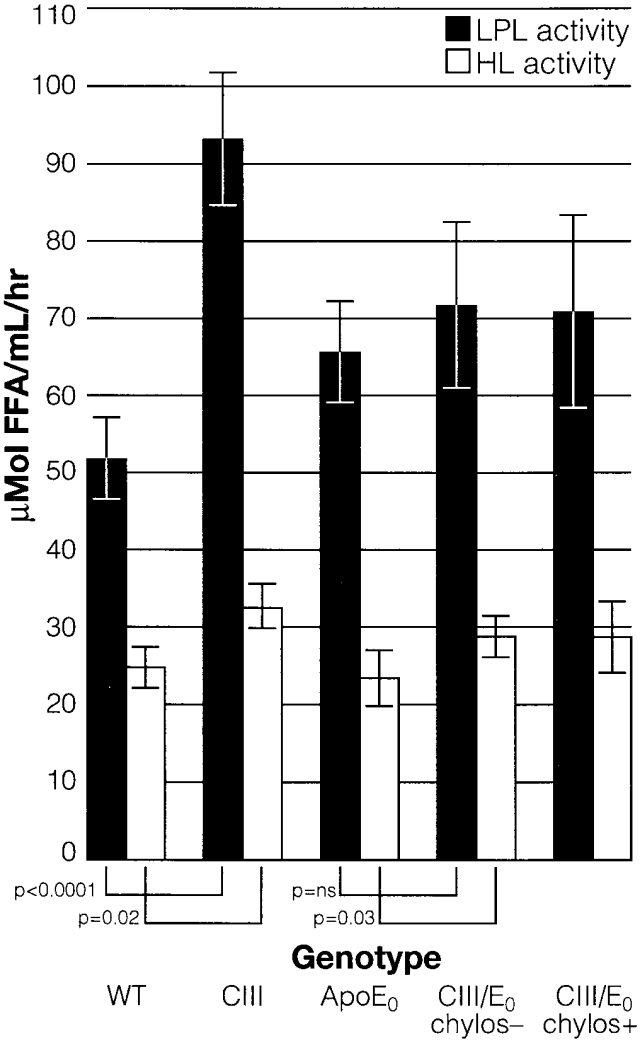

Figure 5. Plasma postheparin LPL and hepatic lipase activities in WT, HuCIII Tg, apoE_0, CIII/E_0 nonlipemic (*chylos*−), and CIII/E_0 lipemic (*chylos*+) mice. The solid bars indicate LPL activity while the hollow bars indicate hepatic lipase activity. Means±SD are displayed.

LPL activation. The activation of mouse LPL was assayed with varying amounts of pooled plasma from WT, apoE_0, HuCIII Tg, and CIII/E_0 mice (Fig. 6). ApoE_0 and, in particular, HuCIII Tg and CIII/E_0 plasma produced decreased maximal LPL activity. Results in individual animals with a low and invariant amount (5 µl) of added plasma (Fig. 7) essentially confirmed these findings. All comparisons were statistically significant versus the WT. Differences between the HuCIII Tg values, the apoE_0 values, and the CIII/E_0 values were not significant. Conclusions were unchanged by correction for the amount of triglycerides added with the plasma sample, which was small.

VLDL glycosaminoglycan binding. VLDL from WT, HuCIII Tg, apoE_0, and CIII/E_0 mice were assessed for binding to heparin-Sepharose, a model for the cell-surface glycosaminoglycan matrix (Fig. 8). Interestingly, binding of apoE_0 VLDL was only mildly reduced versus the WT, and this differ-

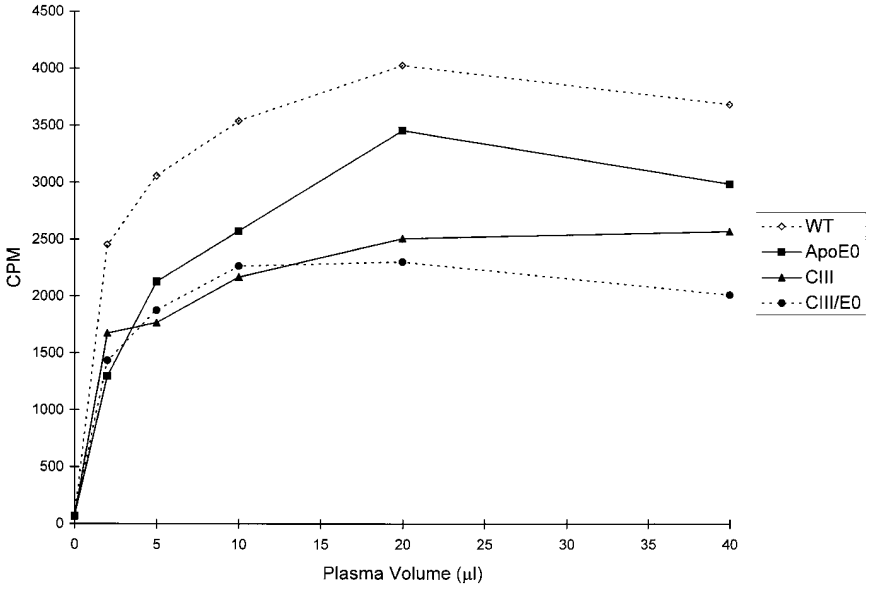

Figure 6. The activation of mouse LPL by varying amounts of pooled plasma from WT, HuCIII Tg, apoE_0, and CIII/E_0 mice. The open diamond symbol with the dotted line represents the WT, the solid square with the uninterrupted line represents apoE_0, the solid triangle with the uninterrupted line represents HuCIII Tg, and the solid circle and dotted line represent CIII/E_0. Decreased activation is noted, in particular related to apoCIII overexpression, with differences already clearly evident upon activation by 5 μl of added plasma.

ence was not statistically significant ($P = 0.12$). A greater fraction of the apoE_0 VLDL binding did not elute with a heparin wash, perhaps indicating higher affinity. The amount of apoE_0 heparin-releasable binding was significantly lower than WT VLDL ($P < 0.0001$). The binding of HuCIII Tg VLDL was markedly reduced, and this difference was very significant for both total and heparin-releasable binding versus both WT ($P < 0.00001$) and apoE_0 VLDL ($P < 0.005$). The binding of CIII/E_0 VLDL was essentially identical to that of HuCIII Tg VLDL.

Atherosclerosis. The observed marked cholesterol elevation in the CIII/E_0 animals was substantially limited to chylomicrons and the lowest density VLDL subfractions. This elevation provided an opportunity to test whether the cholesterol in these fractions is less atherogenic than cholesterol in S_f 20–60, and in LDL (36). We therefore performed a quantitative arteriosclerosis assay in 16 mice from each genotype. The as-

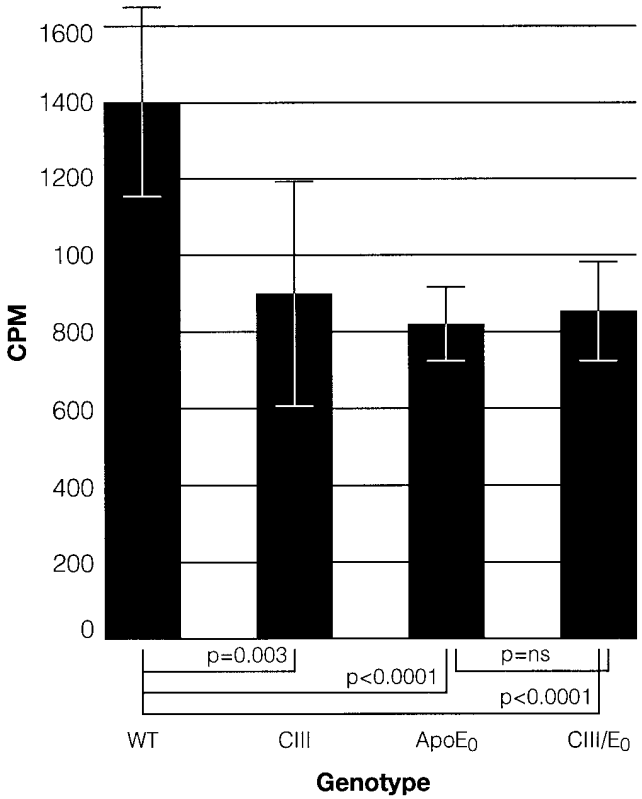

Figure 7. The activation of mouse LPL by 5 μl of plasma from five individual animals each of WT, apoE_0, HuCIII Tg, and CIII/E_0 genotype. Results are presented as mean cpm of ^{3}H-fatty acid±SD.

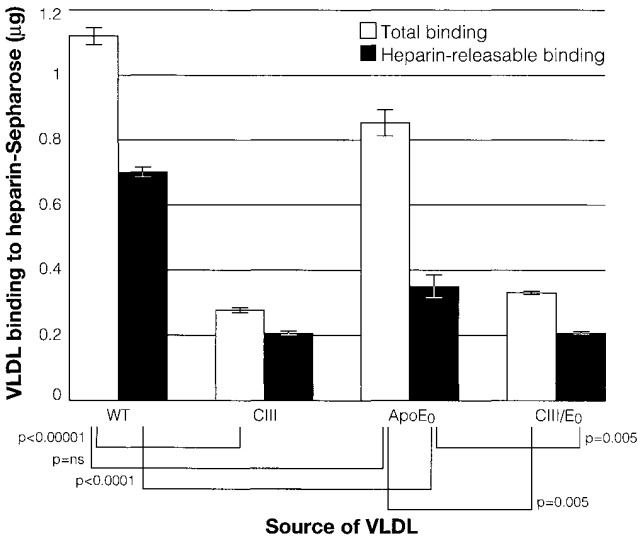

Figure 8. VLDL glycosaminoglycan binding. In vivo–labeled VLDL from WT, HuCIII Tg, apoE_0, and CIII/E_0 mice were incubated with heparin-conjugated agarose, a model for cell-surface glycosaminoglycans. Residual binding after washing was designated total binding, and is displayed by the hollow bars. The portion of total binding eluted by a heparin wash (heparin-releasable binding) is displayed by the solid bars.

say was limited to female animals because of reports of sex-related differences in arteriosclerosis development in mice, with more arteriosclerosis in females (37). Mean lesion areas were the same in the two groups, 48,757±30,590 μm^2 in CIII/E_0 animals vs. 46,381±33,172 μm^2 in apoE_0 animals ($P = 0.83$).

Discussion

In this study we report the effects of apoCIII overexpression in the absence of apoE and, thus, in the absence of any effects attributable to interactions between apoCIII and apoE. CIII/E_0 mice had remarkably elevated triglycerides, well beyond the level reported with this apoCIII transgenic line in the wild-type background (22). A number of animals had levels > 10,000 mg/dl, a level of plasma triglycerides that was not compatible with life in neonatal lipoprotein lipase knockout mice (38). We have not observed plasma triglycerides > 1,000 mg/dl in CIII/E_0 mice under the age of 4 wk, perhaps accounting for the survival of our animals.

The reason for the elevation of triglycerides appears to relate primarily to the decreased lipolysis in vivo which appears evident in earlier work (22, 23). We did not find a significant increase in VLDL triglyceride synthesis, and the relative decrease in the clearance of VLDL triglycerides that we observed in CIII/E_0 versus apoE_0 mice may be an underestimate. Triglycerides in the CIII/E_0 VLDL tracer that we injected into both genotypes may not immediately exhibit the clearance characteristics of triglycerides in apoE_0 VLDL because of slowed exchange of the apoCIII on the lipoprotein tracer with apolipoproteins on apoE_0 TGRL. ApoE may also make a modest contribution to facilitating lipolysis (39). In support of this view, we found evidence of decreased activation of LPL by apoE_0 plasma. ApoE may also displace some apoCIII from TGRL; these effects might be particularly important in the context of apoCIII overexpression. Others have proposed that apoE is an inhibitor of lipolysis (40). The level of apoCIII expression should be about the same in the CIII/E_0 and HuCIII Tg mice, as they were developed from the same human apoCIII overexpressing transgenic line. Any differences between them are substantially attributable to the absence of apoE. The increased triglycerides in the CIII/E_0, where the proposed lipase inhibitor (apoE) would be absent, versus the HuCIII Tg argues against an antilipolytic effect of apoE but does not, by itself, rule out such a role. It does indicate that, in our model, the direct effects of apoCIII in inhibiting lipolysis and the decrease in particle uptake produced by the absence of apoE predominate. We believe that decreased lipolysis because of apoCIII overexpression relates in large part to decreased binding of VLDL to cell-surface glycosaminoglycans. While we did find decreased net activation of mouse LPL by HuCIII Tg and CIII/E_0 plasmas, the decrease in activation was only moderately greater than that seen with plasmas from apoE_0 mice, which were much less hypertriglyceridemic.

The decrease in lipoprotein particle uptake in apoE_0 mice has been documented to be profound, and produces only modest hypertriglyceridemia (25). We do not feel that a further decrease in particle uptake produced by apoCIII overexpression is plausible as the mechanism of the severe hypertriglyceridemia observed in CIII/E_0 versus apoE_0 animals. On the other hand, the absence of apoE-mediated clearance of triglyceride-enriched particles likely accounts for the greater elevation of triglycerides in CIII/E_0 mice than has been reported for the parent human apoCIII transgenic mouse line in a wild-type background (22). We cannot rule out the possibility that apoCIII may also interfere to some degree with the clearance of remnant lipoproteins. This effect may also be at least partly independent of effects on apoE, as has been suggested by in vitro studies (41), and is supported by the increase in IDL+LDL in the CIII/E_0 animals.

The apolipoprotein composition of the particles also reflects the apparent displacement by apoCIII of apoAI. ApoAI is a normal constituent of nascent chylomicrons and, most likely, of VLDL (42). It has been reported to distribute to TGRL in apoE_0 mice, but its absence would not be expected to explain the observed hypertriglyceridemia (40). We did not observe the increased apoAIV in apoE_0 VLDL which was seen by others (25). The ultracentrifugal technique that we used may have led to the artifactual loss of this apolipoprotein.

The role of hypertriglyceridemia in atherosclerosis remains controversial. In univariate analyses of atherosclerotic risk, hypertriglyceridemia has a significant effect, but this may disappear in men and is weakened in women when covariates are taken into account (43). Some have proposed that much of the harmful effect of hypertriglyceridemia may be mediated by the low HDL, small dense LDL, and increased apoB levels associated with most clinical hypertriglyceridemic syndromes. In so-called familial hypertriglyceridemia, where apoB levels are normal, the risk of atherosclerosis is said not to be increased, though this is the subject of controversy (44). The hypothesis that larger TGRL are not directly atherogenic is supported by the long-standing clinical observation that severe hypertriglyceridemia due to genetic LPL deficiency is not associated with atherosclerosis (45), though this hypothesis has recently been called into question (46). These large particles simply may not cross the endothelial cell barrier, as has been described for the large TGRL in diabetic rabbits (36), while smaller VLDL, which have been found within atherosclerotic lesions (47), might have a very different interaction with the vessel wall. Decreased clearance of postprandial TG, which is typically associated with an increase in smaller partially lipolyzed particles, may be independently associated with atherosclerotic risk (48–50). Levels of postprandial apoB48 in the S_f 20–60 density range have specifically been found to be highly predictive of angiographic progression in patients with documented coronary artery disease (51). Interestingly, two recent studies (52, 53) correlated postprandial lipemia with the presence of arteriosclerotic disease in normal weight but not obese men. The obese men had larger TGRL, as indicated by a greater TG/apoB ratio, supporting the view that only the smaller TGRL may be atherogenic.

The predominant increase in large TGRL in the CIII/E_0 mice allowed us to observe the direct effects of hypertriglyceridemia, with triglyceride-enrichment of larger particles, superimposed on an atherogenic (apoE_0) phenotype. The model was complicated by an increase in IDL+LDL and a decrease in HDL in the CIII/E_0 mice. Nevertheless, no increase in atherosclerosis was observed. In contrast, apoCIII overexpression in otherwise wild-type mice on a high cholesterol diet produced a significant, though quantitatively modest, increase in atherosclerosis (54). This increase may have been due to increased IDL+LDL alone. In contrast, the predominant lipoprotein species with atherogenic potential in the CIII/E_0 mice may have been the smaller TGRL species. These species were not increased in the CIII/E_0 mice compared to the apoE_0 mice.

In the context of the severe atherosclerosis produced by these remnant lipoproteins, the observed increase in IDL+LDL in the CIII/E_0 mice may not have been significant. While generalization of these findings to human disease requires caution, the results in this model support the view that a marked increase in large TGRL is not directly proatherogenic.

Acknowledgments

We thank Jan L. Breslow and Alan R. Tall for the use of their facilities for performing the quantitative arteriosclerosis assay, Henry N. Ginsberg and Ira J. Goldberg for advice and critical review of the manuscript, and Teresa Vanni-Reyes, Colleen Ngai, and Kristine Uffelman for technical advice and assistance.

This research was supported by an American Heart Association, New York City Affiliate Investigatorship and Grant-in-Aid to N.S. Shachter.

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