Overexpression of apoC-I in apoE-null mice: severe hypertriglyceridemia due to inhibition of hepatic lipase

Karin Conde-Knape,* André Bensadoun,† Joan H. Sobel,* Jeffrey S. Cohn,§ and Neil S. Shachter[1,*]

Department of Medicine,* Columbia University, 630 W. 168th Street, New York, NY; Divisions of Nutritional and Biological Sciences,† Cornell University, Ithaca, NY; and Hyperlipidemia and Atherosclerosis Research Group,§ Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, Canada

Abstract **Apolipoprotein C-I (apoC-I) has been proposed to act primarily via interference with apoE-mediated lipoprotein uptake. To define actions of apoC-I that are independent of apoE, we crossed a moderately overexpressing human apoC-I transgenic, which possesses a minimal phenotype in the WT background, with the apoE-null mouse. Surprisingly, apoE-null/C-I mice showed much more severe hyperlipidemia than apoE-null littermates in both the fasting and non-fasting states, with an almost doubling of cholesterol, primarily in IDL+LDL, and a marked increase in triglycerides; 3-fold in females to 260 ± 80 mg/dl and 14-fold in males to 1409 ± 594 mg/dl. HDL lipids were not significantly altered but HDL were apoC-I-enriched and apoA-II-depleted. Production rates of VLDL triglyceride were unchanged as was the clearance of post-lipolysis remnant particles. Plasma post-heparin hepatic lipase and lipoprotein lipase levels were undiminished as was the in vitro hydrolysis of apoC-I transgenic VLDL. However, HDL from apoC-I transgenic mice had a marked inhibitory effect on hepatic lipase activity, as did purified apoC-I. LPL activity was minimally affected. Atherosclerosis assay revealed significantly increased atherosclerosis in apoE-null/C-I mice assessed via the en face assay. Inhibition of hepatic lipase may be an important mechanism of the decrease in lipoprotein clearance mediated by apoC-I.**—Conde-Knape, K., A. Bensadoun, J. H. Sobel, J. S. Cohn, and N. S. Shachter. **Overexpression of apoC-I in apoE-null mice: severe hypertriglyceridemia due to inhibition of hepatic lipase. *J. Lipid Res.* 2002.** 43: **2136–2145.**

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Apolipoprotein C-I (apoC-I), a 6.6 kDa plasma protein that is a component of VLDL, IDL, and HDL, has a variety of metabolic functions that continue to emerge. Early investigations emphasized the effect of apoC-I, along with apoC-II and apoC-III, to displace apoE from triglyceride-rich emulsions and lipoproteins, and to thereby interfere indirectly with lipoprotein clearance (1, 2). There was also evidence for a direct inhibitory effect of apoC-I on lipoprotein binding to receptors. Either mixed apoCs or purified apoC-I decreased binding of β-VLDL to a remnant receptor, the LDL receptor-related protein (LRP) (3, 4), and decreased the apoE-mediated binding of human VLDL and IDL to the LDL receptor (LDLR) (5, 6). ApoC-I is an activator of lecithin cholesterol acyl transferase (LCAT), though to a lesser extent than apoA-I (7, 8). ApoC-I has been reported to inhibit plasma phospholipase A2 activity (9). Recently, a previously suggested effect to inhibit cholesteryl ester transfer protein (CETP) (10) has been exhaustively confirmed (11). ApoC-I also was proposed to inhibit lipoprotein lipase (LPL) and hepatic lipase (HL), although the physiological significance of these observations has remained uncertain (12, 13).

The study of genetically modified mice has made an important contribution to current understanding of the metabolic effects of apoC-I. Elevated triglyceride and cholesterol levels in human apoC-I transgenic mice were first reported by Simonet et al. as part of a study establishing the role of DNA sequences 5′ to the apoC-I′ pseudogene in the liver-specific expression of apoC-I and apoE (14). Jong et al. documented an effect of an apoC-I transgene to increase cholesterol and decrease the hepatic uptake of VLDL in APOE*3Leiden transgenic mice (15). Interestingly, triglycerides were much higher in the apoC-I transgenic/APOE*3Leiden mice, but cholesterol levels were not higher than levels in singly transgenic APOE*3Leiden mice. VLDL triglyceride clearance was significantly impaired in intact dually transgenic animals. However, there

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Abbreviations: apo, apolipoprotein; apoE0, apoE gene knockout; Chol, cholesterol; CI, human apoC-I transgenic; E0CI, homozygous for the apoE0 allele and hemizygous for the human apoC-I transgene; HDL-C, HDL cholesterol; HL, hepatic lipase; LDLR, LDL receptor; LRP, LDL receptor-related protein.

[1] To whom correspondence should be addressed.
e-mail: nss5@columbia.edu

was no effect of the apoC-I transgene on VLDL clearance in functionally hepatectomized mice, making it unlikely that apoC-I had a significant in vivo effect on LPL. These results were interpreted as supporting decreased remnant uptake by the liver as the predominant mechanism. However, the functionally hepatectomized mice also had their predominant reservoir of HL excluded from the circulation.

We reported human apoC-I transgenic mice on a WT background, produced using the 154-bp minimal liver-specific enhancer, which we had defined earlier for apoE and apoC-II (16, 17). ApoC-I transgenic mice had combined hyperlipidemia with accumulation of cholesterol-enriched VLDL and IDL+LDL. We documented significantly delayed remnant clearance in support of the postulated mechanism. However, the delay in VLDL TG clearance was much more severe than the delay in the uptake of model cholesterol-enriched remnant particles, an observation that remained unexplained. Jong et al. reported higher-expressing apoC-I transgenic mice that showed a doubling of cholesterol but an 8-fold increase in TG. However, once again, no defect in extrahepatic lipolysis was observed, which was felt to support the proposed liver-uptake mechanism. In addition, a transgenic phenotype was still present in LDLR gene knockout mice, taken as evidence that specific inhibition of LRP-mediated clearance was at least part of this mechanism (18). In subsequent work from this group, apoC-I high expressers were shown to have striking loss of subcutaneous fat along with dermal atrophy and impaired sebum production (19). In a novel action of apoC-I, this appeared to be due to reduced fatty acid metabolism in peripheral tissues, likely due to decreased transport, that was associated with increased sensitivity to insulin but, paradoxically, also with increased glucose levels (20). Additional studies revealed a potent action of increased apoC-I to interfere with lipoprotein clearance via the VLDL receptor (21). ApoC-I gene knockout mice have been reported and had an unremarkable phenotype other than an increased hyperlipidemic response to cholesterol feeding that remains incompletely understood (22).

While pointing to the existence of other mechanisms, these studies have been seen as generally supportive of the prime importance of the inhibition by apoC-I of apoE-mediated lipoprotein uptake pathways. A similar view had been held of apoC-III. However, studies of apoC-III transgenic mice in the apoE-null ($apoE_0$) background revealed a prominent in vivo role for apoC-III as an inhibitor of lipolysis by LPL, in part directly and in part via inhibition of lipoprotein interaction with the cell-surface glycosaminoglycan matrix (23). This work was generally supportive of earlier in vitro studies showing direct inhibition by apoC-III of both LPL (24, 12) and HL (25). In order to elucidate comparable actions of apoC-I that are independent of apoE, we have now performed studies of apoC-I transgenic mice in the $apoE_0$ context. These studies have confirmed the prior observations in transgenic mice of no significant inhibition of LPL but have revealed a prominent inhibition of HL that likely is an important aspect of the role of apoC-I in lipoprotein metabolism.

METHODS

Animals

Human apoC-I transgenic (CI) mice backcrossed for at least six generations to the C57Bl/6 background were used to generate the transgenic/knockout mice (17). ApoE gene knockout ($apoE_0$) mice in the C57Bl/6 background were obtained from the Jackson Laboratory, Bar Harbor, ME (26, 27). CI mice were crossed with the $apoE_0$ mice to generate mice homozygous for the apoE null allele and hemizygous for the human apoC-I transgene (E_0CI). Animals were housed in an approved animal care facility with a 0700 to 1900 period of light. Mice were fed a standard mouse chow diet containing 4.5% fat (10% of calories) and 0.02% cholesterol. Access to food and water was ad libitum except where indicated. Fasting blood was drawn in the afternoon, 6 h after food removal. Non-fasting blood was drawn at 0900. Animals were anesthetized with methoxyflurane for phlebotomy via the retro-orbital plexus and for femoral-vein intravenous injections (NDC 0061-5038-01, Schering-Plough, Union, NJ).

Metabolic chemistry

Cholesterol, triglycerides, and glucose levels were determined in fasting and non-fasting plasma samples using enzymatic kits from Sigma (Cat # 352-20, 339-20, 315-100, Sigma-Aldrich, St. Louis, MO). Free fatty acids (FFA), free cholesterol, and phospholipids were measured using an enzymatic kit from Wako (NEFA-C kit, Cat # 994-75409, Cat # 274-47109 and 990-54001,Wako Chemicals, Richmond, VA). Protein was measured using the BCA protein assay (Cat # 23225, Pierce Chemical, Rockford, IL).

Plasma and lipoprotein concentration of human apoC-I

Human apoC-I concentrations were determined in plasma and lipoproteins of E_0CI mice using an enzyme-linked immunosorbent assay (ELISA) modified from a described apoE ELISA (28). Goat anti-human apoC-I (BioDesign K74110G) was used as the capture antibody at a concentration of 50 μg per plate in 0.1 M sodium carbonate buffer, pH 9. Plates were incubated with the capture antibody overnight at 4°C. After five washes with 0.01 M/l PBS/Tween, plates were blocked at room temperature with 0.25% casein (BDH line/ Gallard Schlessinger #BDH 44023) in PBS for 1 h. After five washes with PBS/Tween, the plates were incubated at room temperature for 2 h with assay samples (in duplicate) that had been diluted 32,000- and 64,000-fold in 0.1% casein/PBS. After five washes with PBS/Tween the plates were incubated at room temperature for 2 h with the tagging antibody (Goat anti-human apoC-I-HRP conjugated, BioDesign #K4110G). After five washes, the plates were incubated for 1 h with orthophenylenediamine (Cat. # P-7288, Sigma-Aldrich) at a concentration of 0.6 mg/ml in 0.051 M/l disodium phosphate, 0.024 M/l sodium citrate, 0.01% hydrogen peroxide, pH 5.0. The reaction was allowed to continue for 1 h and absorbances were read at 415 nm. ApoC-I concentrations were standardized with the use of a human plasma sample of known concentration (gift of Petar Alaupovic) that had been assayed as described (29). Results were further corrected for intraassay variability using a control sample in each plate. The intraassay and interassay coefficients of variation for this assay were 3% and 13% respectively.

Lipoprotein composition

Gel filtration chromatography was performed on 200 μl of pooled mouse plasma (from at least six mice) using two Superose 6 columns in series (FPLC, Pharmacia LKB Biotechnology, Piscataway, NJ). Forty 0.5-ml fractions were collected and assayed for cholesterol and triglycerides as described above. In addition, three distinct pools of plasma from each genotype, each from six mice, were used to isolate lipoprotein fractions. 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 (30). Cholesterol, triglyceride, free cholesterol, phospholipids, and protein concentrations were determined on all fractions.

Subfractionation of triglyceride-rich lipoproteins

Density < 1.006 lipoproteins (Chylomicrons and VLDL) were subfractionated by nonequilibrium density gradient ultracentrifugation as described (31, 32).

Apolipoproteins

VLDL, IDL+LDL, and HDL (10 μg of protein) were fractionated by SDS-PAGE followed by either Coomassie Blue staining or immunoblot. The identity of all the bands identified on the Coomassie Blue gels was confirmed by immunoblot, with the exception of apoA-IV, which was identified solely by molecular weight. Antibodies to mouse apoC (Cat # K23200R, primarily detects apoC-III), mouse apoA-I (Cat # K23001R), and mouse apoA-II (Cat # K23400R) were obtained from BioDesign International (Kennebunk, Maine). Anti-mouse apoC-I was the gift of Karl H. Weisgraber (Gladstone Institute). Antibody to mouse apoB was the gift of Stephen G. Young (Gladstone Institute). Blots were developed with Enhanced Chemiluminescence reagent (ECL; Cat # RPN 2106, Amersham Pharmacia Biotech, Piscataway, NJ), exposed to film, and scanned using a Molecular Dynamics 300a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

VLDL remnant clearance studies

VLDL isolated from $apoE_0$ mice on regular chow were used as described (17). These particles are TG depleted and cholesterol enriched and represent a model for remnant lipoproteins that, due to rapid apolipoprotein exchange, reflect the characteristics of the plasma milieu (17). VLDL apolipoproteins were labeled with ^{125}I using the Iodo-beads technique (Cat # 223240, Pierce Chemical, Rockford, IL). After labeling, an aliquot of VLDL was fractionated by SDS-PAGE and exposed to film to determine the percent radioactivity incorporated into apoB. About 85% of the label was incorporated into apoB. Six mice from each genotype were injected with 1 million counts per min (cpm) of the labeled preparation. Plasma samples were obtained at 30 s and 5, 10, 20, 40, 80, and 120 min after injection. The data (total cpm in plasma) were modeled as described, assuming the value obtained at 30 s as 100% of the injected dose (32).

Triglyceride production rate

VLDL triglyceride secretion rates were determined by inhibiting the catabolism of VLDL by injecting Triton WR1339 followed by serial plasma TG measurements at 0, 0.5, 30, 60, and 90 min, as described (17).

HL and LPL activities

HL activity was determined in pre and post-heparin plasma from six mice of each genotype using an emulsion of [^{3}H]triolein stabilized with guar gum (33). LPL activity was determined in the same plasma samples using [^{3}H]triolein in an Intralipid emulsion, as described (34, 35).

Lipolysis of VLDL by HL and LPL

VLDL from C57Bl/6 mice (WT) as a control, or from C57Bl/6 background apoC-I or apoC-III transgenic mice were used as the substrate. HL hydrolysis of VLDL was performed as described (36). In brief, constant amounts of VLDL (61.9 μg of triglycerides) were incubated with 50 ng of purified rat HL (37) in 60 μl of incubation buffer (0.33 mol/l Tris-HCl, pH 8.3, 1% fatty acid-free BSA, 5 mM $CaCl_2$) brought to a final volume of 200 μl with PBS. Incubations were carried out for 30 min at 37°C. Reactions were terminated by placing the samples on ice. FFA levels were determined immediately using the Wako "NEFA-C" kit (Cat # 994-75409). LPL hydrolysis of VLDL was quantitated as described (38). In brief, 20.1 μg of VLDL triglycerides were incubated for 6 min at 37°C with 14 ng of bovine milk purified LPL (Cat # L-2254, Sigma-Aldrich) in 0.1 M/l Tris pH 8.5 with 2% fatty acid-free BSA in a final volume of 65 μl. The reaction was stopped by addition of 0.1 M/l Tris/0.1% Triton X100 and placing the samples on ice followed by immediate determination of FFA concentrations.

To determine the effect of HDL on the hydrolysis by HL and by LPL of a VLDL substrate, the above assay was performed including varying concentrations of HDL (0 to 30 μg of HDL protein). Two different sources of HDL were used: HDL from WT C57Bl/6 mice and from human apoC-I transgenic mice. The effect of human apoC-I on both assays was assessed similarly, by the addition of 0 to 12 μg of purified apoC-I (Cat # 178459, Calbiochem) to the VLDL substrate. Levels of released FFA were determined as described above.

Atherosclerosis

Seven female mice from each genotype were sacrificed at 6 months of age for the determination of atherosclerotic lesions by the whole-aorta en face assay, the most statistically powerful of the currently standard techniques (39). The whole aorta was carefully removed, placed on a slide and fixed overnight in formaldehyde. After fixation the adventitial fat was removed and the aorta was incised, opened, and stained with Oil Red O. After staining the aortas were scanned and the percentage area containing atheroma (Oil Red O-positive) was quantitated (40).

Statistical analysis

Statistical analysis was by Student's t-test. Two-tailed P values of 0.05 were considered statistically significant. Due to skewing, triglyceride levels were log-transformed for statistical analyses.

RESULTS

Plasma lipids

We crossed a moderately overexpressing human apoC-I transgene into the $apoE_0$ background. E_0CI mice showed much more severe hyperlipidemia than $apoE_0$ littermates in both the fasting and non-fasting states, with an almost doubling of cholesterol and an 8-fold increase in TG. When the data were analyzed by sex, E_0CI males had a 14-fold increase in TG compared to $apoE_0$ males, with a doubling of cholesterol, while E_0CI females had a 3-fold increase in TG compared to $apoE_0$ females, again with cholesterol levels that almost doubled (**Table 1**). Subsequent studies were conducted in males, except where indicated.

TABLE 1. Fasting plasma lipids (mg/dl) in $apoE_0$ and E_0CI mice, by sex

	CHOL	TG	GLU
$ApoE_0$-females	621 ± 131	80 ± 21	179 ± 42
E_0CI-females	1077 ± 184	260 ± 80	174 ± 38
P	< 0.0001	< 0.0001	NS
$ApoE_0$-males	716 ± 149	137 ± 61	176 ± 33
E_0CI-males	1508 ± 198	1409 ± 594	215 ± 72
P	<0.0001	<0.0001	0.03

CHOL, total cholesterol; HDL-C, HDL cholesterol; TG, triglycerides; GLU, glucose. Plasma lipids from $apoE_0$ (23 females and 19 males) and E_0CI (20 females and 21 males).

TABLE 2. Lipoprotein fraction composition in apoE$_0$ and E$_0$CI mice

	FC	CE	TG	PL	PRO	(CE+TG)/(FC+PL+PRO)
			mg/dl (%)			
VLDL						
ApoE$_0$	55 ± 6	420 ± 158	27 ± 11	90 ± 19	60 ± 9	2.15 ± 0.46
	(9 ± 2)	(63 ± 7)	(4 ± 2)	(14 ± 1)	(9 ± 1)	
E$_0$CI	176 ± 34	1156 ± 343	550 ± 212	183 ± 15	127 ± 14	3.47 ± 0.94
	(8 ± 3)	(52 ± 3)	(24 ± 5)	(9 ± 2)	(6 ± 1)	
P	0.003	0.02	0.01	0.002	0.002	NS
	(NS)	(NS)	(0.001)	(0.006)	(0.01)	
IDL+LDL						
ApoE$_0$	22 ± 2	53 ± 12	3 ± 1	34 ± 4	37 ± 2	0.59 ± 0.07
	(15 ± 1)	(35 ± 3)	(2 ± 1)	(23 ± 1)	(25 ± 2)	
E$_0$CI	33 ± 4	152 ± 13	24 ± 3	66 ± 6	57 ± 3	1.13 ± 0.12
	(10 ± 1)	(46 ± 2)	(7 ± 1)	(20 ± 1)	(17 ± 1)	
P	0.01	0.0006	0.0003	0.001	0.0008	0.002
	(0.002)	(0.01)	(0.0008)	(0.02)	(0.001)	
HDL						
ApoE$_0$	15 ± 4	29 ± 9	2 ± 2	23 ± 5	95 ± 10	0.22 ± 0.26
	(9 ± 1)	(17 ± 3)	(1 ± 1)	(14 ± 2)	(59 ± 6)	
E$_0$CI	11 ± 2	20 ± 8	16 ± 9	17 ± 5	72 ± 29	0.38 ± 0.26
	(8 ± 2)	(15 ± 6)	(12 ± 7)	(12 ± 4)	(53 ± 18)	
P	NS	NS	NS	NS	NS	NS

Lipoprotein composition in apoE$_0$ and E$_0$CI mice. Plasma from at least six mice was pooled for lipoprotein isolation. Values (mg/dl) represent the mean ± SD for each parameter in three distinct pools from each genotype. Values in parentheses represent percentages (or *P* values based on analysis of percentages). NS, not significant.

Plasma lipoproteins

The composition of the ultracentrifugally isolated lipoprotein fractions is shown in **Table 2**. VLDL from E$_0$CI mice had an increase in the absolute amounts of all measured lipoprotein constituents. On a relative basis, VLDL from E$_0$CI mice was triglyceride enriched and, correspondingly, cholesterol-depleted compared to apoE$_0$ mice. The IDL+LDL fraction from E$_0$CI mice was also increased in absolute amount and moderately TG-enriched. HDL from E$_0$CI mice appeared to have more TG compared to apoE$_0$ mice, but this difference was not statistically significant. IDL+LDL from E$_0$CI mice had a significantly higher core constituent (cholesteryl ester + TG) to surface constituent (free cholesterol + phospholipids + protein) ratio, with a similar trend for VLDL and HDL. These differences, indicative of larger particles, are more consistent with decreased lipolysis than decreased remnant clearance.

Gel filtration chromatography of whole plasma confirmed that E$_0$CI mice had increased cholesterol and triglycerides, both in the VLDL and IDL+LDL fractions, when compared to apoE$_0$ mice (**Fig. 1**).

Density < 1.006 g/ml lipoproteins (chylomicrons and VLDL) were subfractionated by nonequilibrium density ultracentrifugation (**Table 3** and **Fig. 2**). Results in the Svedberg coefficient of flotation (S$_f$) 100-chylomicron subfraction showed a significant increase in the absolute cholesterol and TG content of this subfraction in E$_0$CI mice. The absolute quantity of all constituents was also significantly increased in the S$_f$ 60–100 subfraction from E$_0$CI mice, which was also significantly TG-enriched compared to the same fraction from apoE$_0$ mice, although apparently to a lesser extent than the S$_f$ 100-chylomicron subfraction. The S$_f$ 20–60 subfraction in E$_0$CI mice was increased, on an absolute basis, to the least extent and was significantly cholesterol-depleted to the benefit of TG. Overall, 57% of total VLDL TG was in the S$_f$ 100-chylomicron fraction in E$_0$CI mice compared to 44% in apoE$_0$ mice, while only 10% of VLDL TG was in the S$_f$ 20–60 subfraction in E$_0$CI mice compared to 19% in apoE$_0$. This disproportionate increase in larger particles again suggested

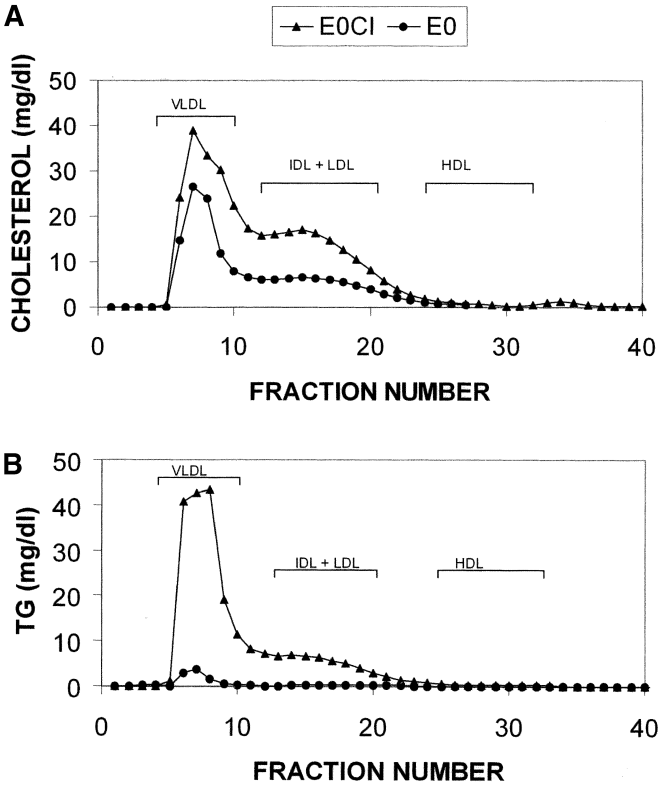

Fig. 1. Lipoprotein fractionation by gel filtration chromatography (FPLC) of pooled plasma from E$_0$CI and apoE$_0$ mice. A: Cholesterol distribution. B: Triglyceride distribution.

TABLE 3. Subfractionation of density < 1.006 g/ml lipoproteins

	CHOL	TG	PL	PRO
		mg/dl (%)		
S_f 100-Chy				
$ApoE_0$	49 ± 8 (58 ± 3)	7 ± 1 (8 ± 1)	16 ± 8 (18 ± 5)	12 ± 2 (15 ± 1)
E_0CI	91 ± 39 (41 ± 0)	69 ± 36 (30 ± 3)	28 ± 8 (13 ± 2)	33 ± 11 (15 ± 1)
P	NS (0.014)	NS (0.01)	NS	NS
S_f 60-100				
$ApoE_0$	89 ± 4 (65 ± 4)	6 ± 0 (5 ± 0)	17 ± 5 (12 ± 4)	25 ± 0 (18 ± 0)
E_0CI	120 ± 1 (51 ± 1)	41 ± 2 (17 ± 1)	33 ± 1 (14 ± 0)	43 ± 5 (18 ± 2)
P	0.007 (0.03)	0.002 (0.005)	0.05 (NS)	0.03 (NS)
S_f 20-60				
$ApoE_0$	73 ± 1 (60 ± 0)	3 ± 1 (2 ± 1)	23 ± 1 (19 ± 1)	23 ± 0 (19 ± 0)
E_0CI	95 ± 12 (54 ± 0)	12 ± 7 (7 ± 3)	33 ± 1 (19 ± 2)	36 ± 3 (21 ± 1)
P	NS (0.002)	NS	0.01 (NS)	0.02 (NS)

Subfraction composition from $apoE_0$ and E_0CI mice. Values (mg/dl) represent the mean ± SD for each parameter from three distinct pools for each genotype. Values in parentheses represent percentages (or *P* values based on analysis of percentages).

an important effect of apoC-I overexpression on lipolysis, not on lipoprotein particle uptake.

Apolipoproteins

HDL apolipoproteins are known to transfer to VLDL in $apoE_0$ animals (27); this transfer appears to be blocked by overexpression of apoC-I. SDS-PAGE/Coomassie Blue staining of VLDL and IDL+LDL from E_0CI mice showed much more apoB, more apoC-I, and less apoA-I and apoA-IV than the corresponding fractions from $apoE_0$ mice (**Fig. 3**). This was confirmed by immunoblot, which revealed, in addition, decreased apoA-II in E_0CI VLDL (**Fig. 4A**). HDL from E_0CI mice had somewhat less apoA-I content but strikingly less apoA-II than $apoE_0$ HDL (Fig. 4B). Overall, the apoA-I/apoA-II ratio was significantly lower in VLDL from E_0CI mice but significantly higher in HDL (Fig. 4C).

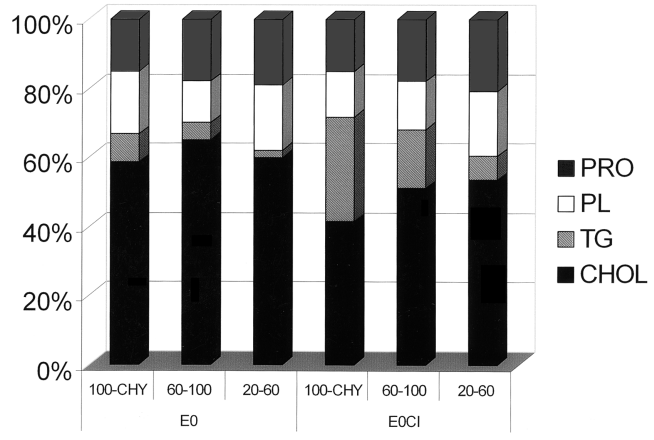

Fig. 2. Composition of VLDL density subfractions. PRO, protein; PL, phospholipids; TG, triglycerides; CHOL, cholesterol.

VLDL remnant clearance

To investigate the mechanism underlying the doubling of cholesterol observed in E_0CI mice, we determined the clearance of post-lipolysis remnant-like lipoproteins obtained from $apoE_0$ mice (**Fig. 5**). There was no difference between E_0CI and $apoE_0$ mice in the rate of disappearance of these particles, arguing against any direct effect of apoC-I on post-lipolysis remnant clearance in these animals.

VLDL triglyceride production studies

To investigate the possibility of increased production of VLDL TG as the mechanism responsible for increased TG in E_0CI mice, we examined the changes in plasma TG after the injection of Triton WR1339, an inhibitor of VLDL lipolysis. As shown in **Fig. 6**, there was no difference between E_0CI and $apoE_0$ mice in the rate of VLDL production, as assessed by the post-Triton increase in plasma TG.

Post-heparin lipase activities

Given the absence of an effect on VLDL production or particulate uptake, we examined the mice for a defect in lipolytic clearance pathways. Levels of HL and LPL were measured in E_0CI, $apoE_0$, C57Bl/6 (WT), and human apoC-I transgenic mice as their post-heparin activities in plasma. As shown in **Fig. 7A**, E_0CI and $apoE_0$ mice had higher LPL activity compared to WT mice. HL activity was also increased in E_0CI mice compared to $apoE_0$ (Fig. 7B), as we have seen in other hyperlipidemic models, perhaps due to increased stabilization of the circulating lipase (23, 41).

Lipolysis of VLDL

We then examined the properties of VLDL as a substrate for both LPL and HL (**Fig. 8**). VLDL isolated from three different transgenic genotypes (C-I, C-III, and WT)

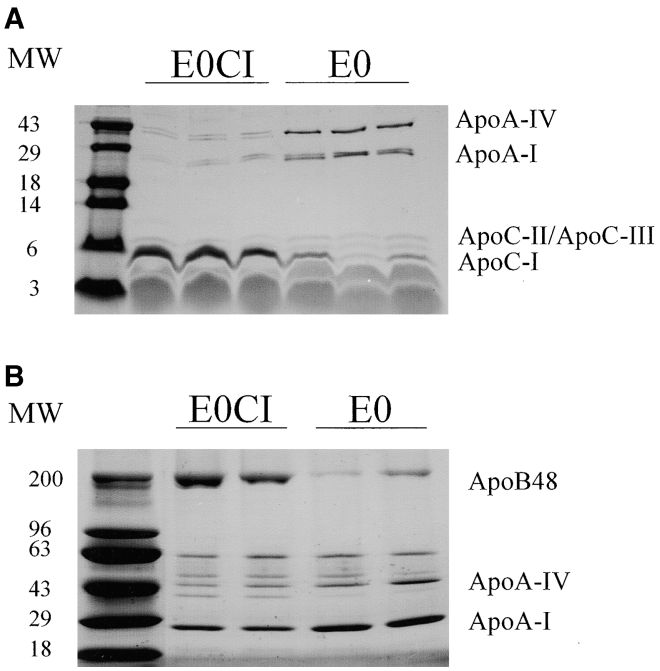

Fig. 3. SDS-PAGE of apolipoproteins from E_0CI and $apoE_0$ mice, stained with Coomassie Blue. A: VLDL. B: LDL.

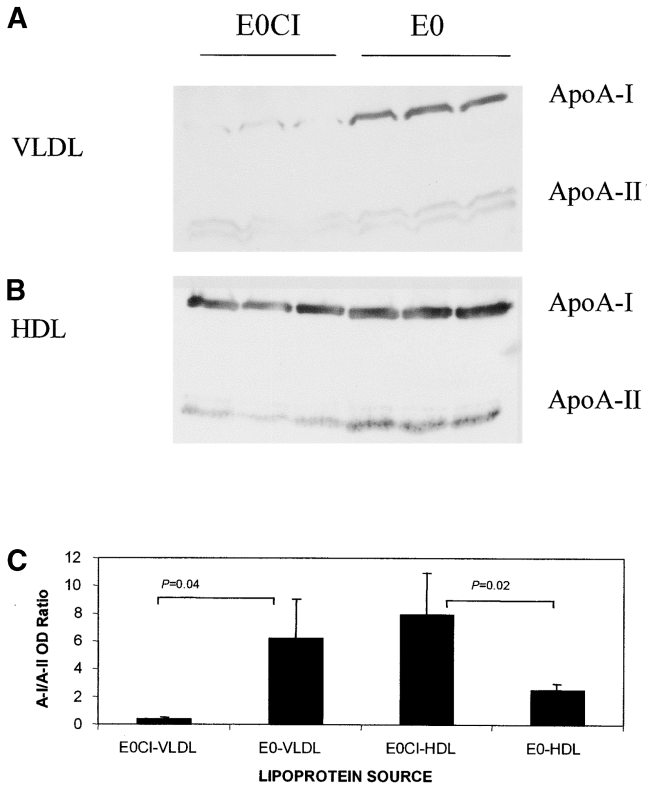

Fig. 4. Immunoblots for apoA-I and apoA-II in VLDL and HDL from E_0CI and $apoE_0$ mice. A: VLDL. B: HDL. C: ApoA-I/apoA-II ratios in VLDL and HDL from E_0CI and $apoE_0$ mice.

were used as substrates for both LPL and HL. $ApoE_0$ animals could not be used in these experiments ($apoE_0$ VLDL has very little TG). VLDL from CIII mice were hydrolyzed poorly by both LPL and HL, consistent with the described inhibitory effect of apoC-III on both LPL and HL activities (12, 24, 25). There was no evident defect in the hydrolysis by either enzyme of VLDL from CI mice.

VLDL lipolysis: effect of HDL and of apoC-I

To replicate a more physiological lipolytic environment, in which apolipoproteins transferred from HDL play an important role (42), we repeated the assay in the presence of HDL isolated from either CI or WT mice. HDL isolated from CI mice had less apoA-I and apoA-II compared to that obtained from WT mice, presumably due to in-

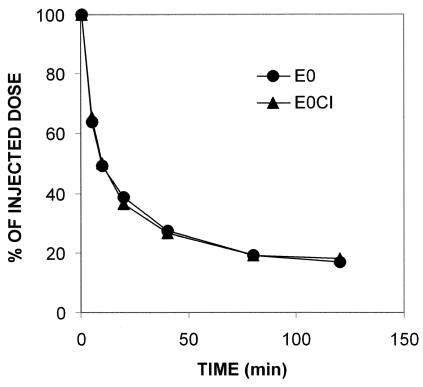

Fig. 5. Clearance of post-lipolysis lipoprotein "remnants." E_0CI and $apoE_0$ mice were injected with 1,000,000 cpm of ^{125}I-labeled $apoE_0$ VLDL. Plasma samples were obtained at time intervals ranging from 30 s to 120 min.

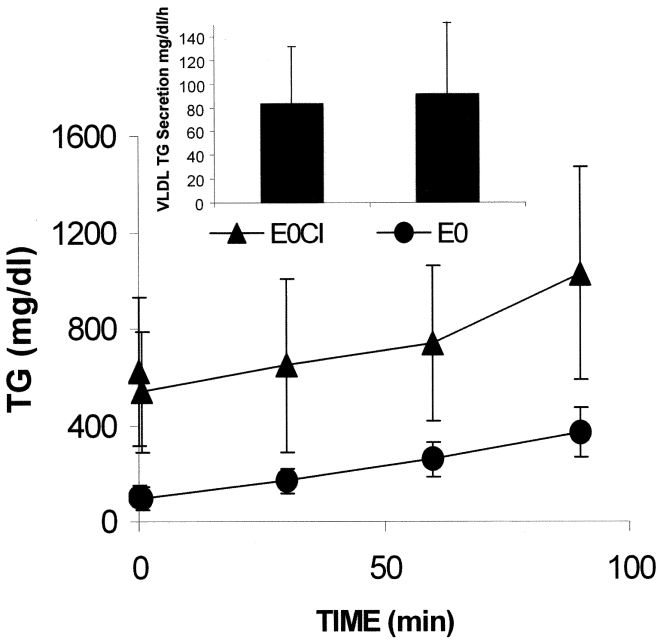

Fig. 6. Secretion of VLDL TG. Triglyceride levels over time in plasma of E_0CI and $apoE_0$ mice after the injection of Triton WR1339 to block VLDL lipolysis. The insert shows the calculated VLDL TG secretion rates in mg/dl/h.

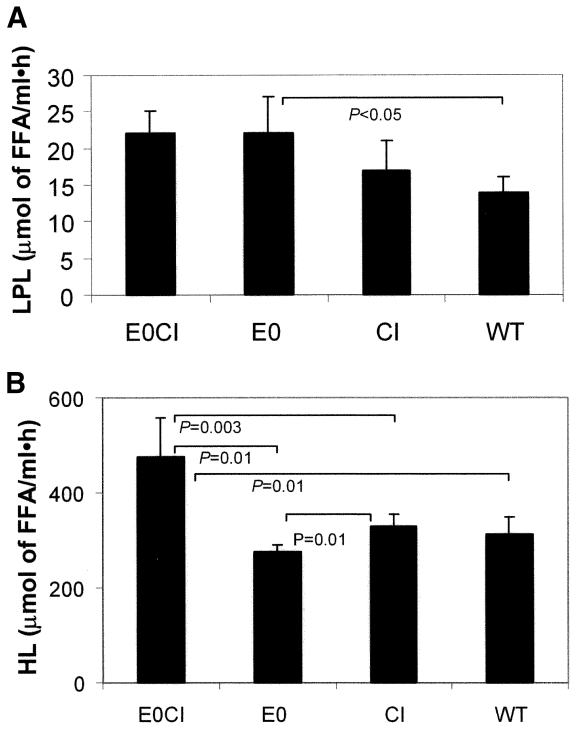

Fig. 7. LPL and HL activities in postheparin plasma from E_0CI, $apoE_0$, apo C-I transgenic (CI), and C57Bl/6 (WT) mice. A: LPL activity. B: HL activity.

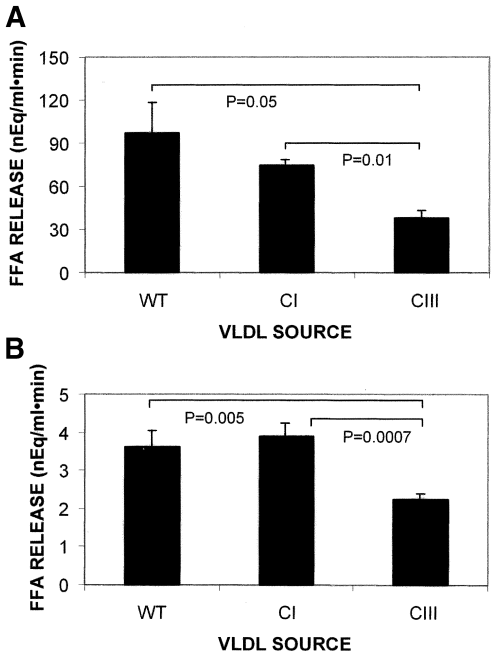

Fig. 8. VLDL lipolysis. VLDL were isolated from C57Bl/6 (WT), apoC-I transgenic (CI) mice, and apoC-III transgenic (CIII) mice. A: FFA released by LPL. B: FFA released by HL.

creased apoC-I (**Fig. 9A**). The apoA-I/apoA-II ratio appeared higher in the CI HDL, as was noted in the E_0CI HDL, but this difference was not statistically significant (Fig. 9B). HDL from either WT or CI mice had no effect on the hydrolysis of WT VLDL by LPL (**Fig. 10A**). In contrast, HDL inhibited VLDL hydrolysis by HL in a dose dependent manner (Fig. 10B). Importantly, HDL isolated from CI mice was a much stronger inhibitor of HL hydrolysis at all concentrations tested.

We then repeated the experiment with the addition of purified apoC-I in place of HDL to see if this apolipoprotein could be accounting for the inhibitory effect of HDL. The addition of apoC-I to the VLDL hydrolysis reaction

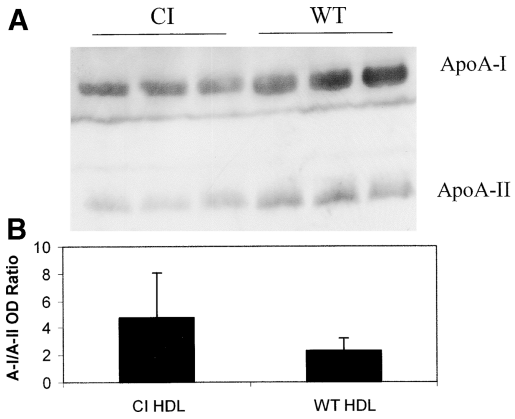

Fig. 9. ApoA-I and apoA-II in HDL. A: Western blot of apoA-I and apoA-II in HDL from apoC-I transgenic (CI) mice and C57Bl/6 (WT) mice. B: ApoA-I/apoA-II ratios in HDL isolated from CI and WT mice.

resulted in a slight inhibition of LPL activity that was only evident at high concentrations (8 μg and 12 μg) (**Fig. 11A**). In contrast, addition of apoC-I resulted in a dose dependent inhibition of HL hydrolysis that was already significant at 0.5 μg and that completely abolished HL activity at 8 μg (Fig. 11B). The VLDL and HDL isolated from CI mice had concentrations of 5.1 and 9.3 mg/dl of human apoC-I, respectively, accounting for 6% of VLDL and 4.5% of HDL total protein.

Atherosclerosis

Because of the proposed protective effect of HL deficiency on atherosclerosis in the apoE_0 context, a quantitative atherosclerosis assay was performed to determine the effect on atherosclerosis of the hypercholesterolemia associated with the apoC-I transgene (43). This revealed significantly more atherosclerosis in E_0CI mice (27 ± 7% of surface area vs. 19 ± 5% for apoE_0, $P = 0.02$), consistent with the increase in IDL+LDL that we observed in these animals.

DISCUSSION

In the present study we report the effects of apoC-I overexpression in the absence of apoE and, thus, in the absence of any effects attributable to interactions between apoC-I and apoE. E_0CI mice had increased VLDL and

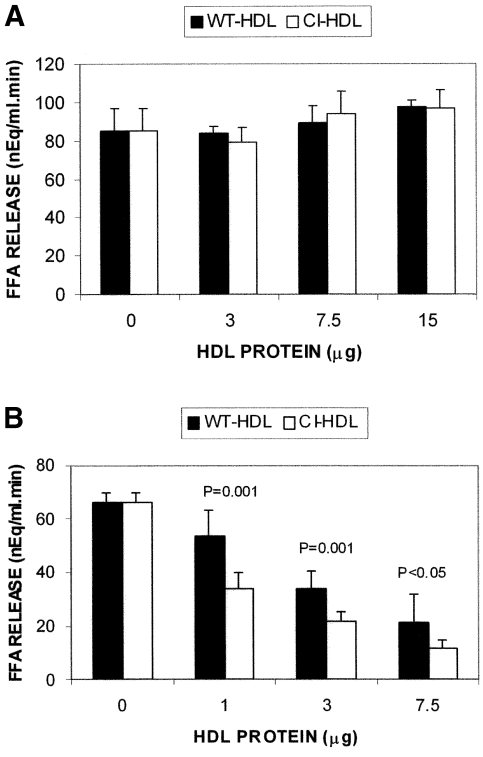

Fig. 10. Inhibition of VLDL lipolysis by HDL. VLDL isolated from C57Bl/6 (WT) mice were incubated with varying concentrations of HDL isolated from either C57Bl/6 (WT) mice or apoC-I transgenic mice (CI). A: Free fatty acids (FFA) released by LPL. B: FFA released by HL.

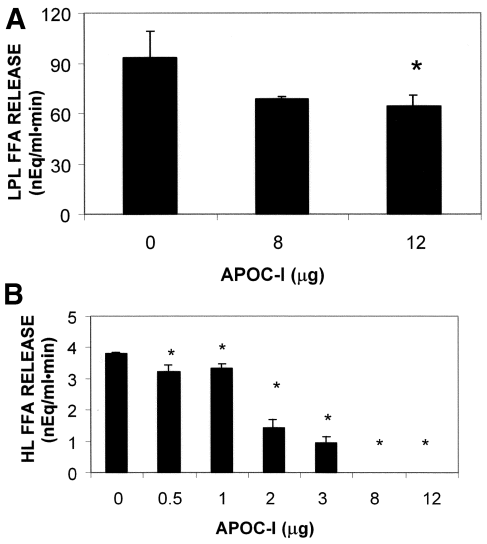

Fig. 11. Inhibition of VLDL lipolysis by purified human apoC-I. VLDL isolated from C57Bl/6 mice (WT) were incubated with varying concentrations of purified human apo C-I protein. A: FFA released by LPL. B: FFA released by HL. $*P < 0.02$ versus control (0 μg).

IDL+LDL leading to an 8-fold increase in TG and a doubling of already very high levels of cholesterol. There was a prominent gender difference in the effect on TG, as is commonly seen with metabolic interventions in mice (44). The observed changes were well beyond those reported with this apoC-I transgenic line in the wild type background, where hemizygotes have a very modest phenotype (17). The reason for this elevation of lipids appears to be the inhibition of HL that was proposed by an early in vitro study (13) and that is implicit, in retrospect, in prior transgenic mouse studies. Those studies documented decreased clearance of VLDL TG out of proportion to the remnant clearance defect and without any evidence for decreased lipolysis by LPL (15, 17, 18). There was no evidence in the current study for alternative mechanisms such as effects on VLDL secretion rates. Based on the lack of change in the phospholipid composition of the lipoproteins, there was no evident inhibitory effect on the phospholipase activity of HL, despite the described effect of apoC-I as a phospholipase A2 inhibitor (9). Lipoproteins that have been acted upon by phospholipase are rapidly cleared and may not be contributing appreciably to the assayed pool (45, 46). However, no effect on postlipolysis remnant clearance was detected. Additionally, there were no effects on body weight, subcutaneous fat, dermal integrity, or fatty acid levels with the moderately expressing transgene used in this study (data not shown). A modest effect of the transgene to increase plasma glucose achieved statistical significance in the fasting state in both males and females, confirming a prior report (20).

In the present study, this effect on HL was mediated only by HDL and by added isolated apoC-I but not by VLDL, perhaps due to differences in the surface packing or conformation of the apoC-I molecules in VLDL, where protein is a much smaller fraction of particle mass. Interestingly, inhibition of both LPL and HL was evident with apoC-III-transgenic VLDL, consistent with the prior literature, and perhaps indicating different mechanisms for the HL inhibition mediated by these two apolipoproteins. ApoC-I in HDL was more potent than purified apoC-I as an HL inhibitor, again likely due to conformation effects. In the absence of lipid, purified apolipoproteins aggregate and would be expected to have very different biochemical potency (47). In contrast, the inhibition of LPL by apoC-I that was also observed in the early literature does not appear to be physiologically significant (12, 15, 17, 18). The similar effects of transgenic HDL and purified apoC-I on HL as compared to LPL provide further support that apoC-I itself was the active principal. To exclude extraneous sources of variability, the mice used were age-matched and essentially genetically identical other than the presence or absence of the transgene. Moreover, based on compositional analysis of the particles, secondary effects of the transgene on HDL composition would appear unlikely as an explanation. In particular, apoA-II, an inhibitor of HL, decreased along with an apparent increase in the apoA-I/apoA-II ratio (48–52). Consistent with this observation, increased catalytically inactive HL has been shown to accelerate the clearance of apoA-II (57). Decreased activity of hepatic lipase would also be expected to increase the retention of apoA-I in HDL particles, which would contribute to the observed increase in the apoA-I/apoA-II ratio (53).

The E_0CI phenotype does not match the phenotype of a genetically engineered HL null allele in the apoE_0 context, where only modestly increased plasma TG were observed (43). However, human HL deficiency, whether due to decreased expression or secretion of an inactive protein, is associated with combined hyperlipidemia with a prominent hypertriglyceridemic component that is similar to the findings in apoC-I transgenic mice (54–56). The presence of some HL protein in the human cases could emphasize a lipolytic defect rather than a clearance defect by permitting the preservation of HL's cholesteryl ester uptake and "bridging" functions, as has been shown in mice injected with a recombinant adenovirus coding for an inactive HL variant (57). In the knockout mouse the reverse situation might obtain: the presence of a nonexpressing HL allele might allow the high levels of LPL that are present in the mouse to occupy hepatic sites and take over some of HL's hydrolytic but not its other roles, as the authors of the initial report of this mouse speculated (58). If apoC-I is functioning as an HL inhibitor in the mouse, it may be creating a situation that is similar to HL deficiency in humans: the enzyme would still occupy its hepatic sites and thus its hydrolytic role would not be compensated for by LPL. Indeed, inactivation of HL by antibody injection in rats or cynomolgus monkeys produces a phenotype similar to that observed in HL-deficient humans (59, 60).

The HL knockout/apoE_0 mouse also exhibited decreased atherosclerosis, the opposite of our observations. The differences in the lipoprotein phenotypes of these models may provide the explanation for this discordance.

Decreased atherosclerosis in HL knockout/apoE_0 mice was attributed to an increase in the low levels of HDL that are normally present in apoE_0 mice (43). No such increase in HDL was observed in our mice, consistent with the reported observation that, in contrast to HL absence, increased catalytically inactive HL had a minimal effect on HDL concentrations (61). Our results are also consistent with atherosclerosis assay findings in other mouse models, where HL overexpression led to a decrease in atherosclerosis (62).

The extent of contribution of this novel action of apoC-I to the physiological effect of apoC-I when in the presence of apoE will require further investigation. However, based on our findings, we would propose that the classically described effect of apoC-I to interfere with apoE-mediated lipoprotein remnant clearance may be due, in significant measure, to interference with actions of HL that contribute to entry into the apoE-mediated clearance pathway (63–68).

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