Decreased HDL cholesterol levels but normal lipid absorption, growth, and feeding behavior in apolipoprotein A-IV knockout mice

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Abstract To determine the physiological role of apolipoprotein (apo) A-IV, knockout mice were created by gene targeting in embryonic stem cells. In apoA-IV knockout mice, plasma cholesterol and triglyceride levels were reduced 25% and 44%, respectively, compared with controls. These changes were accounted for by decreased high density (HDL) and very low density lipoprotein (VLDL) levels, respectively, and metabolic studies indicated increased HDL-cholesteryl ester (CE) fractional catabolic rate (FCR) and reduced VLDL transport rate (TR), respectively. ApoA-IV knockout mice had greater than 70% reductions in both hepatic and intestinal apoC-III RNA levels and a similar reduction in the plasma apoC-III level. Complementation analysis, via crossbreeding of a mouse apoC-III transgene onto both the normal and apoA-IV knockout backgrounds, clearly demonstrated that the low triglyceride (VLDL) level in the apoA-IV knockout mice was due to alterations in apoC-III and not apoA-IV. ApoA-IV knockout mice had normal growth, feeding behavior, and lipid absorption, except male mice showed increased food intake in the 2 h after an 18-h fast, suggesting that under some circumstances apoA-IV might serve as a satiety factor. In summary, studies in apoA-IV-induced mutant mice have demonstrated a role for apoA-IV in increasing HDL cholesterol by inhibiting HDL cholesteryl ester FCR yet argue against the apolipoprotein as an overall important mediator of lipid absorption/metabolism.—**Weinstock, P. H., C. L. Bisgaier, T. Hayek, K. Aalto-Setälä, E. Sehayek, L. Wu, P. Sheiffele, M. Merkel, A.D. Essenburg, and J. L. Breslow.** Decreased HDL cholesterol levels but normal lipid absorption, growth, and feeding behavior in apolipoprotein A-IV knockout mice. *J. Lipid Res.* 1997. **38:** 1782–1794.

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Apolipoprotein (apo) A-IV is a 46 kD protein (394 amino acids) associated primarily with chylomicrons, HDL, and the lipoprotein-free fraction of plasma (1–9). The apoA-IV gene resides in a 15 kb gene complex on human chromosome 11 (mouse chromosome 9) along with apoA-I and apoC-III (10, 11). From 5′ to 3′ the gene order is apoA-I, apoC-III, and apoA-IV with the apoC-III gene transcribed from the opposite DNA strand (2). In spite of relatively high apoA-IV levels in plasma (15 to 37 mg/dl), the in vivo function of this apolipoprotein is not known. Due to its almost exclusive pattern of expression in the small intestine, apoA-IV has been postulated to play a role in lipid absorption and chylomicron secretion (12.) After fat feeding, plasma apoA-IV levels rise as does intestinal apoA-IV synthesis (4, 7, 13–15). By comparing patients with apoA-I/C-III/A-IV deficiency (16, 17) with subjects deficient in only apoA-I and apoC-III (18, 19), it has been suggested that apoA-IV might play a role in the absorption of fat soluble vitamins, such as vitamins A and E. Other proposed roles for apoA-IV include cofactor activity for LCAT (20, 21), as an interstitial carrier of cholesterol in reverse cholesterol transport (22), and as a satiety signal to reduce further food intake after fat ingestion (23–25).

In an attempt to reveal apoA-IV function, transgenic mice have been created that express human apoA-IV mainly in intestine and have dramatically elevated plasma apoA-IV levels (26). These mice had normal fasting cholesterol and triglyceride levels, but in the fed state had elevated triglyceride levels, due to reduced

Abbreviations: apo, apolipoprotein; FCR, fractional catabolic rate; TR, transport rate; VLDL, very low density lipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; ES, embryonic stem cell; neo, neomycin resistance gene; -C, cholesterol; -CE, cholesteryl ester; WTD, Western-type diet; RA, retinyl acetate; RP, retinyl palmitate; TG, triglyceride; PAGE, polyacrylamide gel electrophoresis; AIV-2, wild type mouse; AIV-1, heterozygote apoA-IV knockout mouse; AIV-0, homozygote apoA-IV knockout mouse; FFA, free fatty acid.

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clearance of VLDL particles. Cholesterol, triglyceride, and fat-soluble vitamin absorption were normal in the human apoA-IV transgenic mice, as were weight gain, amount of food consumed, and feeding behavior. Thus, even very high levels of apoA-IV expression in mice, which were certainly above the physiological range, did not provide important insights into apoA-IV function in the body.

One interpretation of these negative results in human apoA-IV transgenic mice is that normal levels of apoA-IV are sufficient to carry out its function(s) and that an apoA-IV deficiency state would have to be created to discern the true physiological role of this apolipoprotein. Therefore, in the current study, apoA-IV-deficient mice were created by homologous recombination in embryonic stem cells. In the fasted and fed state the knockout mice had approximately 44% decreased triglyceride levels and 22% decreased HDL cholesterol levels. The decrease in triglycerides was shown to be due to decreased expression of the neighboring apoC-III gene in the knockout mice and not to apoA-IV deficiency. The HDL cholesterol reduction was accompanied by normal apoA-I levels and was due to increased HDL cholesteryl ester fractional catabolic rate. The apoA-IV-deficient mice had normal lipid absorption, weight gain, and food consumption, with a suggestion of increased ingestion in male, but not female, knockout mice after an 18-h fast.

METHODS

Generation of apoA-IV-deficient mice

pPW26.1, a replacement-type targeting vector, was constructed with isogenic S129/J DNA. First, the 1.1 kb neomycin resistance (neo) gene sequence from pMCI-NEO (27) was ligated into pSP72 (Stratagene Inc.). Then the 7 kb Xhol-Notl fragment extending from 1 kb 5′ to 8 kb 5′ of the apoA-IV gene, including exons 1 and 2 of the apoC-III gene, was ligated upstream of neo. Finally, the 1.5 kb Xba1-Sac1 fragment extending from the 1st intron into the 3rd exon of the apoA-IV gene was ligated downstream of neo. After linearization with Not I, 10 μg of targeting vector pPW26.1 was electroporated (BRL Cell-Porator) into 16×10^6 ES cells in 0.9 ml of ES cell medium (28) at 200 V and 800 μF. Stable integrants underwent positive selection in 200, 400, or 800 μg/ml G418 and colonies were picked into 96-well plates 10 days after electroporation. After expansion to 24-well plates, clones were trypsinized with half frozen down and half expanded for genomic. Southern blot analysis. The correctly targeted clones were injected into C57BL/6J host blastocysts and 10–20 embryos were transferred into the uterine horn of (C57BL/6J × CBA/J)F1 surrogate mothers (29). All resulting chimeric animals were back-crossed to C57BL/6J mice and germline transmission was scored by coat pigment. Heterozygous knockout mice were identified by genomic Southern blotting of tail tip DNA and were interbred to generate homozygotes.

Genomic Southern blot analysis

Emryonic stem cell (ES) cell DNA was isolated by digesting cells in lysis buffer (1% SDS, 625 μg/ml proteinase K, 100 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA) at 55°C overnight and spooling genomic DNA after precipitation in ethanol. Tail tip DNA was prepared by rocking tail tips in lysis buffer (30) at 55°C overnight and spooling genomic DNA after ethanol precipitation. To screen, ES cell and mouse DNAs were digested with EcoRI and analyzed by genomic Southern blot according to Walsh, Ito, and Breslow (30)

Northern blotting

Liver and small intestine were dissected from 2–3 fasted males of each genotype and total RNA was extracted by the method of Chomczynski and Sacchi (31). Total RNA (20 μg) was denatured by 2.2 M formaldehyde and 50% formamide at 68°C for 15 min prior to being run on a 1.0% agarose gel with formaldehyde. RNA was transferred to a nylon membrane (Hybond-N, Amersham Corp) by capillary blotting and hybridized to probes at 65°C degrees under standard conditions. The mouse apoA-I and C-III probes were both 188 nt riboprobes (32). The mouse apoA-IV probe was the same as that used for Southern blotting (3′ flanking DNA probe A). Mouse B-actin riboprobe (pTRI-B Actin-mouse, Cat # 7423, Ambion Inc.) was used to normalize for equal loading of RNA between lanes.

Apolipoprotein analysis

Mouse apoA-IV protein levels were measured by Western blot analysis using a rabbit anti-rat apoA-IV that cross-reacts with the mouse protein (33). Mouse apoA-I and apoC-III were measured by rocket immunoelectrophoresis using cross-reactive goat anti-rat apoC-III (a gift from Dr. P. Dolphin) and sheep anti-mouse apoA-I and antibodies, respectively.

Lipid and lipoprotein analysis

Adult mouse blood was obtained from mice in the morning after they had normal access to food (fed samples) and in the evening after they had fasted 8 h during the day (fasted samples). Total plasma triglyceride and cholesterol were determined enzymatically using commercial kits (no. 236691 and no. 126012, respectively;

Boehringer Mannheim Corp. Indianapolis, IN). In previous work, mouse free glycerol was measured to be minimal and therefore was not corrected for in the present study. Lipoprotein cholesterol contents (profiles) were determined by on-line post-column analysis of Superose-6 gel-filtered plasma (HPGC) as previously described (26).

HDL and apoA-I turnover

HDL turnover studies were carried out using doubly labeled HDL as described previously (34). Briefly, HDL-CE was labeled with [^{3}H]cholesteryl-oleoyl ether previously dissolved in intralipid and transferred into HDL by the addition of purified CETP from d >1.25 g/ml rabbit plasma. Mouse apoA-I was purified and radiolabeled with ^{125}I by the Billheimer modification of the McFarlane method as previously described (34). The specific activity of ^{125}I-labeled apoA-I was approximately 200 cpm/ng. Prior to injection, 2–4 μg of ^{125}I-labeled apoA-I was mixed with mouse [^{3}H]cholesteryl-oleyl ether-labeled HDL (100,000–200,000 dpm). Before experiments, blood was drawn from fed mice for determination of baseline plasma HDL-C and apoA-I levels. Animals of each genotype were injected intravenously with doubly labeled HDL and bled at 10 min, 90 min, 3 h, 8 h, and 24 h. Twenty μl plasma from each time point was counted in a gamma counter to measure ^{125}I-labeled apoA-I radioactivity. Another aliquot of plasma (40 μl) was extracted with hexane and [^{3}H]cholesterol oleoyl ether radioactivity was measured by liquid scintillation. Fractional catabolic rates (FCR) for apoA-I and HDL-CE were calculated from the plasma decay curves of ^{125}I-labeled apoA-I and [^{3}H]cholesteryl oleoyl ether assuming a two-pool model as in the Matthews method (35).

In vivo production and clearance of labeled VLDL

Control and knockout mice were injected intravenously with [^{3}H]palmitate (200 μCi) and bled 45 min later. VLDL (d < 1.006 g/ml) was isolated by ultracentrifugation. Greater than 70% of radiolabeled material was associated with triglycerides, as assessed by thin-layer chromatography followed by liquid scintillation counting. Clearance of radiolabeled VLDL (3×10^5 dpm/mouse) was determined in control and knockout mice maintained for 2 weeks on a Western-type diet (WTD). Control and knockout mice were injected with [^{3}H]VLDL harvested from mice of the same genotype. Recipient mice received an intravenous bolus of [^{3}H]VLDL and were bled at 2, 5, 10, 20, 40, 75, and 120 min for determination of serum radioactivity. Results are expressed as the per cent of the 2-minute radioactivity. To determine hepatic triglyceride production, control and knockout mice were anesthetized and treated with Triton WR 1339 (500 mg/kg i.v.) to block lipolysis (36). Mice were then injected with [^{3}H]glycerol (100 μCi i.v.) and blood samples were obtained at 20, 30, 60, 90, and 120 min. Lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (37). Triglycerides were separated by thin-layer chromatography on silica gel G (Analtech Inc., Newark, DE) in hexane–diethyl ether–acetic acid 82:16:1 (vol/vol/vol), and radioactivity was determined by liquid scintillation counting.

Growth and feeding behavior

Growth curves were produced by weighing control and knockout 2.5-month-old female mice once per week for 8 weeks. Ad lib food intake and feeding behavior after 18-h food deprivation was studied in three groups of animals. Studies 1, 2, and 3 involved wild type and knockout mice of 129/b6, 129/b6, and 129 strains, respectively. All animals were between 4 and 5 months of age. First, animals were caged individually and allowed to accommodate to their environment for 1 week. For 3 to 4 weeks animals were then monitored once per week for food intake and weight gain. Calculations were made to determine grams gained per day as well as total gain over the observation period. The average weight over the observation period was determined by dividing the total weight gained by two and adding that to the initial weight at the start of the experiment. At the end of the observation period mice were deprived of food for 18 h beginning at 6 PM. Food intake was then measured by providing animals with cups of food (6 g/cup) for 30, 60, and 120 min. Calculations were made to determine grams consumed/grams body weight at each of the time points and are expressed as averages ± standard deviation.

Vitamin A-fat tolerance test

Control and apoA-IV knockout mice were fasted overnight and then given an intragastric bolus of retinyl palmitate (RP, 3000 U) in corn oil (100 μl), followed by 150 μl corn oil, followed by 100 μl air. Mice were bled before and 1, 2, 4, and 10 h after vitamin A administration. Retinyl acetate (RA) internal standard was added to serum samples before lipid extraction (38). Samples were resuspended in toluene (15 μl) and analyzed by reverse-phase HPLC (Beckman Instruments, Inc. Fullerton, CA) using 2 ml/min methanol as the mobile phase (39). Peak area of retinyl palmitate was normalized to that of retinyl acetate.

Fat tolerance test

Mice were studied both in the absence and presence of i.v. Triton WR1339. Male control and apoA-IV knockout mice were fasted for 16 h followed by intragastric

administration of 1 ml corn oil. These mice were bled at 0, 0.25, 0.5, 1, 2, and 4 h for determination of plasma triglycerides. Female control and apoA-IV knockout mice were fasted for 16 h, treated with Triton WR 1339 (500 mg/kg iv.), and 10 min later given 0.5 ml corn oil intragastrically. These mice were bled at 0, 0.25, 0.5, 1, 1.5, 4, 13, and 24 h for determination of plasma triglycerides.

Cholesterol absorption

Cholesterol absorption was determined in control and knockout littermates maintained on chow diet by the "single isotopic meal feeding" method (40). Mice received an intragastric bolus of [^{3}H]sitostanol (1.67 μCi, American Radiolabeled Chemical, Inc.) and [^{14}C]cholesterol (0.67 μCi, Amersham Corp.) in 100 μl safflower (MCT) oil. After 5 days, feces were collected, dried overnight at 55°C, and extracted (1 g) with chloroform–methanol 1:1 followed by KOH–ether–water. The top phase was then counted. The percent absorption was calculated from the ratio of ^{14}C/^{3}H in the dosing mixture minus the ^{14}C/^{3}H ratio in the feces divided by the ratio of ^{14}C/^{3}H in the dosing mixture.

SDS PAGE

Six 5-month-old mice from each group were bled in the fed state and plasmas were pooled by genotype. Superose 6 gel filtration chromatography fractions were collected and an aliquot from each was assayed by standard enzymatic assay (kit no. 704036; Boehringer Mannheim Corp. Indianapolis, IN) to determine cholesterol profiles. Fractions forming the VLDL peak were combined, concentrated by speed vacuum, and subjected to 4–15% gradient SDS PAGE. Gels were stained with Coomassie blue R-250.

RESULTS

Targeted disruption of the apoA-IV gene

Figure 1A shows the apoA-I/C-III/A-IV locus on mouse chromosome 9. A vector (*pPW26.1*, Fig. 1B) was constructed to replace 1.1 kb 5′ of the apoA-IV gene, exon 1, and part of intron 1 with the neo gene (neo) (Fig. 1C). Homologous recombination was documented by the presence of a new mutant 4.8 kb Eco RI band along with the wild type 14.4 kb Eco RI band (Figs. 1D and 1E) when ES cell DNA was probed with apoA-IV exon 3 sequences (Fig. 1F), thus indicating the desired incorporation of a new Eco RI site residing within the neo gene. One-in-thirteen picked clones proved to be correctly targeted and were expanded and injected into blastocysts. Of the 40 mice generated, 80% were chimeric and 25% were entirely agouti. Eight of the later were mated with C57Bl6 mice, and all gave rise to germ line transmission as indicated by the presence of agouti pups, and confirmed by Southern blot analysis of tail tip DNA. Interbreeding apoA-IV heterozygous knockout mice resulted in the correct Mendelian ratio of wild type (AIV-2) to heterozygote (AIV-1) to knockout (AIV-0) mice (Fig. 1H). Northern blot hybridization of total RNA from mice of all three genotypes with the DNA probe from apoA-IV exon 3 revealed a gene dosage-dependent reduction in intestinal apoA-IV RNA, with none of the mice showing hepatic apoA-IV RNA (Fig. 1I). Hybridization of the same RNA with an apoA-I probe showed a 50% reduction in intestinal apoA-I expression with no change in liver expression. Western blot analysis using a rabbit anti-rat apoA-IV antibody showed a gene dosage-dependent decrease in plasma apoA-IV with none detectable in the homozygous knockout mice (Fig. 1J).

Lipid and lipoprotein levels in mice lacking apoA-IV

As shown in **Table 1,** on a chow diet (4.5% fat, 0.02% cholesterol) in the fed state, A-IV knockout mouse triglyceride and cholesterol levels were reduced 45% and 23%, respectively, when compared to littermate controls, with almost identical reductions observed in the fasted state. This was due to 61% and 23% reductions in VLDL and HDL (as measured by their cholesterol levels), respectively. Similar decreases in VLDL and HDL levels were also observed in apoA-IV knockout mice maintained on Western-type (21% fat, 0.15% cholesterol) and sucrose (chow plus 10% sucrose in the drinking water) diets as shown by the lipoprotein patterns in **Fig. 2.** On the Western type diet the apoA-IV knockout mice failed to elevate VLDL levels when going from the fasted to the fed state as observed in control mice. This was also seen, albeit less strikingly, in animals maintained on the sucrose diet. Heterozygous apoA-IV knockout mice showed an intermediate phenotype (data not shown). Female mice showed similar trends, but the differences between knockout and control mice were less pronounced (data not shown).

HDL metabolism

To rule out inadvertent alterations in the expression of neighboring gene apoA-I as the cause of reduced HDL seen in apoA-IV knockouts, apoA-I tissue RNA and plasma protein levels were measured. Though intestinal apoA-I RNA was reduced 50% in apoA-IV knockout mice, liver expression was normal (Fig. 1I) and this pattern of expression was sufficient to produce normal circulating amounts of apoA-I protein **(Table 2).** Turnover studies were then performed with HDL

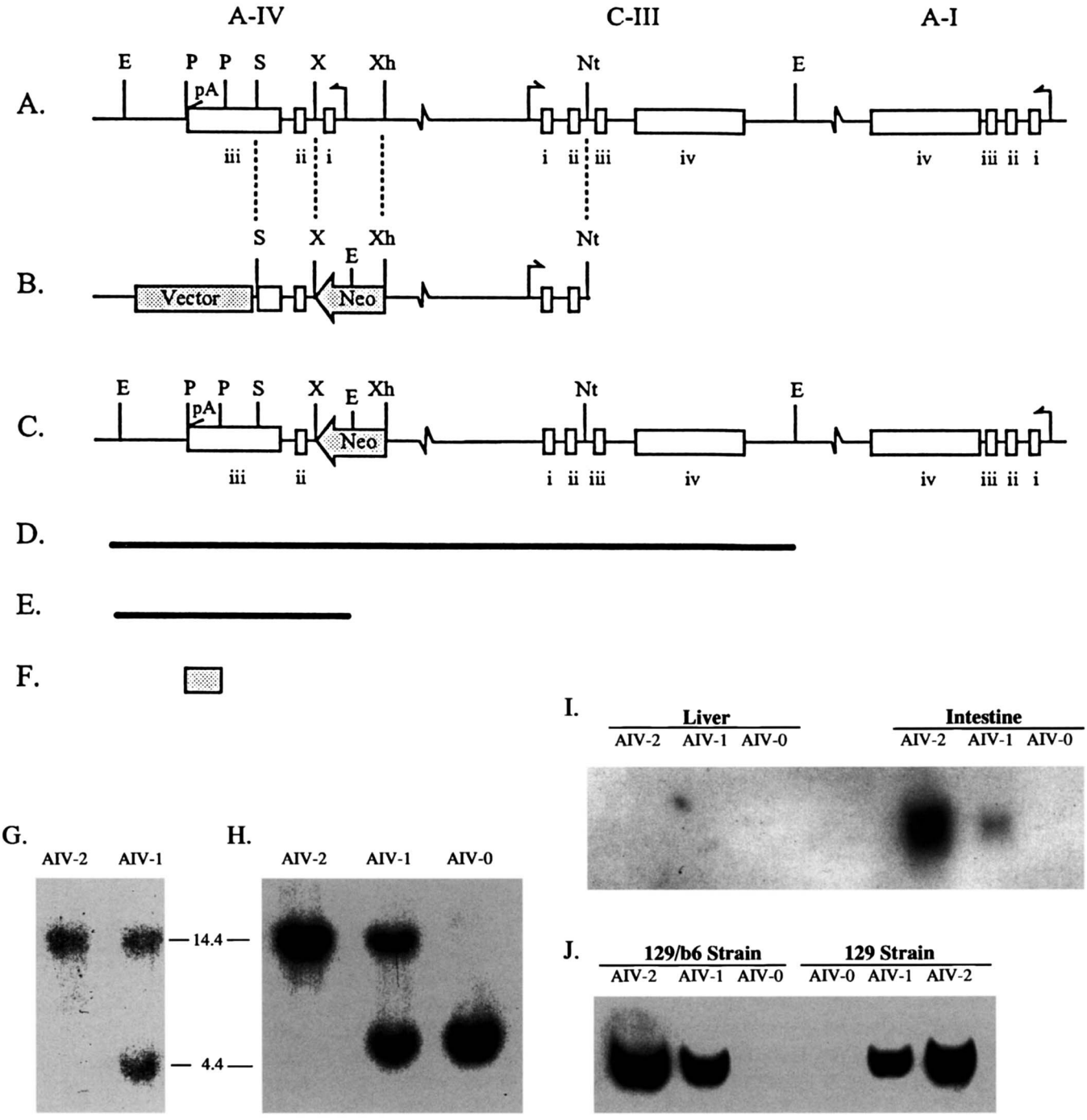


Fig. 1. Targeted disruption of the apoA-IV gene. Line (A) represents the map of the apoA-IV-C-III-A-I gene locus on mouse chromosome 9. Open boxes denote exons labeled with roman numerals. Restriction endonuclease sites used for cloning and screening are shown (E = EcoRI, P = PstI, S = SacI, X = XbaI, Xh = XhoI, Nt = NotI). pA, polyadenylation site. Bent arrows indicate transcription start sites. Line (B) represents the targeting vector pPW26.1 which contains 7 and 1.1 kb of 5′ and 3′ apoA-IV DNA sequences, respectively. The hatched box and arrow denote the backbone plasmid pSP72 (Stratagene, Inc.) and the neomycin resistance gene (neo) from pMC1NEO, respectively. A strategy of "polyadenylation capture" was used as the neomycin resistance gene (neo), when properly recombined, utilized the apoA-IV polyadenylation sequence. Line (C) illustrates the predicted organization of the ApoA-IV locus after homologous recombination. Lines (D) and (E) represent the predicted fragment lengths after EcoRI digestion of apoA-IV wild type (14.4 kb) and induced mutant (4.4 kb) alleles. Box (F) represents the flanking probe used for confirming the targeting event. (G and H) Southern blot analysis of ES cell and mouse tail tip DNA, respectively. DNA was digested with restriction endonuclease EcoRI and probed with the flanking probe. Fragment size is reported in kb. AIV-2, normal ES cell and mouse DNA; AIV-1, targeted ES cell DNA and heterozygous deficient mice; AIV-0, homozygous deficient mice. (I) Northern blot analysis of total RNA from liver and intestine of fasted male mice. (J) Western blot analysis of plasma from fasted male mice of outbred 129/bl6 strain and inbred 129 strain probed with rabbit anti-rat apoA-IV antibody. The 46 kd apoA-IV band is indicated.

TABLE 1. Plasma lipids of apoA-IV knockout mice

	n	TG	TC	VLDL-C	LDL-C	HDL-C
			mg/dl			
Fed						
AIV-2	10	101 ± 30	98 ± 19	4 ± 1	13 ± 3	85 ± 11
AIV-0	9	56 ± 22[a]	75 ± 21[b]	2 ± 0[c]	13 ± 3	66 ± 18[b]
Fasted						
AIV-2	10	74 ± 30	97 ± 15	4 ± 2	12 ± 2	86 ± 13
AIV-0	9	39 ± 7[a]	76 ± 16[a]	1 ± 0[a]	11 ± 4	68 ± 15[b]

Analysis of 2- to 3-month-old male mice. All data represent means ± SD. *P* values were determined by Student's *t*-test.
[a] $P < 0.008$ versus AIV-2.
[b] $P < 0.03$ versus AIV-2.
[c] $P < 0.0001$ versus AIV-2.

doubly labeled with ^{125}I-labeled apoA-I and [$_3$H]cholesterol-oleoyl ether to determine the metabolic basis for the decrease in HDL cholesterol observed in the apoA-IV knockout mice. Despite normal plasma apoA-I levels, apoA-IV knockout mice had a 27% increase in HDL-CE fractional catabolic rate (FCR) compared to controls, which entirely accounted for the reduction in HDL cholesterol levels. There was no significant change in HDL-CE transport rate (TR) in the knockout mice.

VLDL metabolism

VLDL turnover studies were performed to determine the metabolic cause of the reduced triglycerides and VLDL in the apoA-IV knockout mice. Endogenously labeled VLDL from knockout and control mice were injected into Western diet-fed mice of the same genotype. In spite of a 40% reduction in plasma triglyceride levels in the apoA-IV knockout mice compared to controls, both groups of mice had nearly identical rates of loss of plasma radioactivity, as shown in **Fig. 3A.** This suggests that the decrease in triglyceride and VLDL levels in the apoA-IV knockout mice is due to decreased TR rather than increased FCR. In another study, radiolabeled VLDL was harvested from apoA-IV knockout mice and injected into both chow-fed apoA-IV knockout and control mice with the same result (data not shown). To confirm that a primary decrease in TR was responsible for the reduced VLDL seen in AIV-0 mice, mice were pre-treated with Triton WR 1339 to block clearance, and the time course of incorporation of injected [^{3}H]glycerol into plasma triglycerides was determined. As shown in **Fig. 3B,** the rate of appearance was

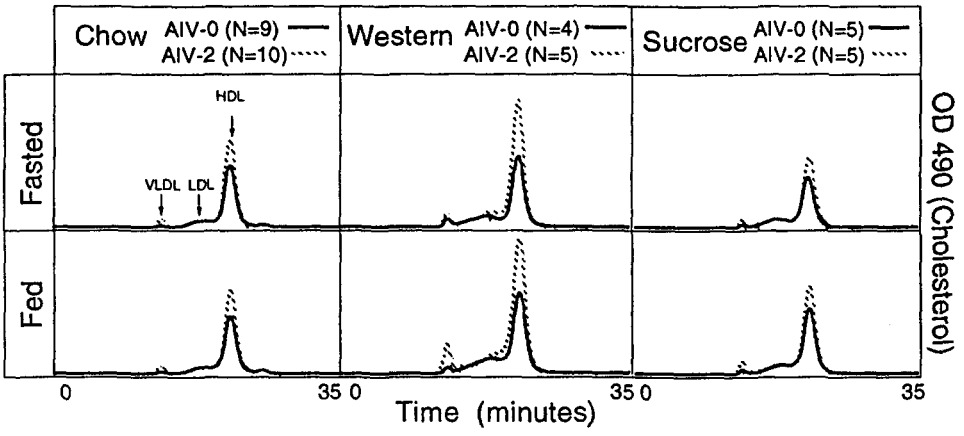


Fig. 2. Lipoprotein cholesterol profiles of apoA-IV knockout and control mice on chow and lipogenic diets. Total lipoprotein cholesterol profiles were determined by on line post-column analysis of Superose 6 gel-filtered mouse serum as previously described (26). Plasma obtained from both fed (8 AM) and fasted (8 h during the day) 3-month-old male animals maintained on chow (4.5% fat, 0.02% cholesterol), Western type diet (Western, 21% fat, 0.15% cholesterol) and sucrose (sucrose, chow plus 10% sucrose in the drinking water) diets were pooled and analyzed. Bold and dotted lines denote AIV-0 and AIV-2 mice, respectively. All profiles are in the same scale.

TABLE 2. HDL metabolism in apoA-IV knockout mice

	n	HDL-CE	HDL-CE FCR	HDL-CE TR	ApoA-I	ApoA-I FCR	ApoA-I TR
		mg/dl	*pools/h*	*U*	*mg/dl*	*pools/h*	*U*
AIV-2	20	65 ± 16	0.125 ± 0.019	7.7 ± 1.7	250 ± 50	0.083 ± 0.014	21.5 ± 7.0
AIV-0	16	40 ± 13[a]	0.158 ± 0.025[b]	6.2 ± 2.5	236 ± 96	0.091 ± 0.014	20.7 ± 9.6

Three-month-old male and female animals were analyzed. Data represent means ± SD. *P* values were determined by Student's *t*-test.
[a] $P < 0.0001$ versus AIV-2.
[b] $P < 0.001$ versus AIV-2.

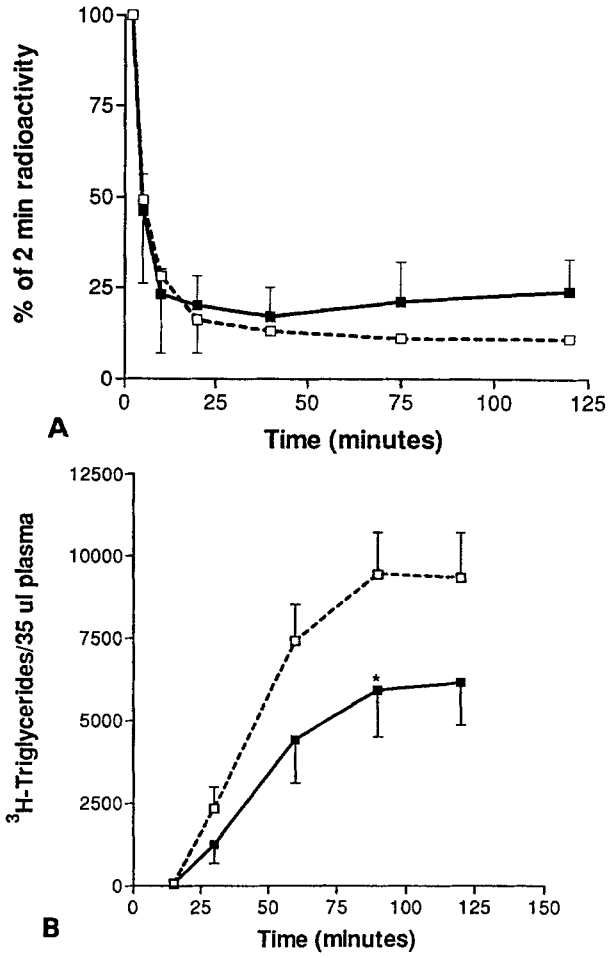


Fig. 3. VLDL triglyceride clearance and production in apoA-IV knockout mice. VLDL clearance was studied in four control (□) and four homozygous knockout (■) mice using [^{3}H]triglyceride VLDL metabolically labeled in knockout and control mice as described in Methods. [^{3}H]triglyceride VLDL was intravenously administered to mice and serum radioactivity was determined at intervals up to 120 min. (A) Radioactive decay curves for ^{3}H-labeled VLDL triglycerides in control and knockout mice. (B) Hepatic triglyceride production was determined in eight control and eight homozygous apoA-IV knockout mice as described in Methods. Mice were treated with Triton WR 1339 (to block lipolysis) and then intravenously injected with [^{3}H]glycerol. Serum triglyceride radioactivity was determined up to 2 h after injection. Data represent mean ± SD; $*P < 0.09$ by Student's *t*-test.

decreased in apoA-IV knockout compared to control mice ($P < .09$). Serum FFA levels were found to be normal in apoA-IV knockout mice in both the fed and fasted states (data not shown), eliminating the possibility that decreased FFA delivery to liver might be the underlying cause of their reduced VLDL TR.

Expression of apoC-III in apoA-IV knockout mice

The expression of the neighboring apoC-III gene was next examined in the apoA-IV knockout mouse. As shown in **Fig. 4A,** Northern blot analysis of total RNA with a mouse apoC-III riboprobe revealed greater than a 70% reduction in apoC-III RNA in both the liver and intestine of apoA-IV knockout mice compared with controls. As shown in Fig. 4B, rocket immunoelectrophoresis utilizing an antibody to apoC-III reveals a similar decrease in plasma apoC-III levels. As apoC-III levels are a determinant of plasma triglyceride levels, it was uncertain whether the decreased triglyceride levels in the apoA-IV knockout mice were due to the lack of apoA-IV or the decreased apoC-III. To examine these alternatives, a mouse apoC-III transgene (41) was bred onto the apoA-IV knockout and control genetic backgrounds and in genotypically identical groups of mice plasma triglyceride and apoC-III concentrations were compared. As shown in Fig. 4C, triglyceride levels correlated with apoC-III concentrations across all groups ($R^2 = .95$, $P < .0001$). Thus the absence of apoA-IV did not exert an independent effect on plasma triglyceride levels, and the decrease in triglycerides in the apoA-IV knockout mice is due to the associated decrease in apoC-III gene expression.

Weight gain, food intake, and feeding behavior

Weight gain, food intake, and feeding behavior were next assessed in apoA-IV knockout mice. Female apoA-IV knockout mice and controls were weighed twice a week from 2 to 4 months of age, and beginning weights and weight gain did not differ, as shown in **Fig. 5A.** In another series of experiments, initial weight, weight gain, and ad lib food intake were monitored beginning at 4 to 5 months of age in male 129/b6 (studies 1 and 2) and inbred 129 strain (study 3) mice for 21 to 30 days. As shown in **Table 3,** no differences were observed among the groups. To specifically investigate whether apoA-IV plays a role in signaling satiety, food intake over a 2-h time period after an 18-h fast was monitored in male apoA-IV-deficient and control mice. As shown in Fig. 5B, inbred129 male apoA-IV knockout mice had significantly increased food intake and, although not significant, 129/b6 male apoA-IV knockout mice showed a similar but not significant trend. Female mice did not show this difference (data not shown).

Dietary fat absorption in apoA-IV knockout mice

Intestinal lipid absorption in apoA-IV knockout mice was assessed by several methods. In the vitamin A-fat tolerance test, after an intragastric bolus of vitamin A, plasma levels of retinyl palmitate peaked at 2 h in both apoA-IV knockout and control mice with a nonsignificant trend towards lower levels in apoA-IV knockout mice during the first 4 h, as shown in **Fig. 6A.** Next, plasma triglyceride levels were measured after an intragastric bolus of corn oil without and with pretreatment with Triton WR1339 to block plasma triglyceride clear-

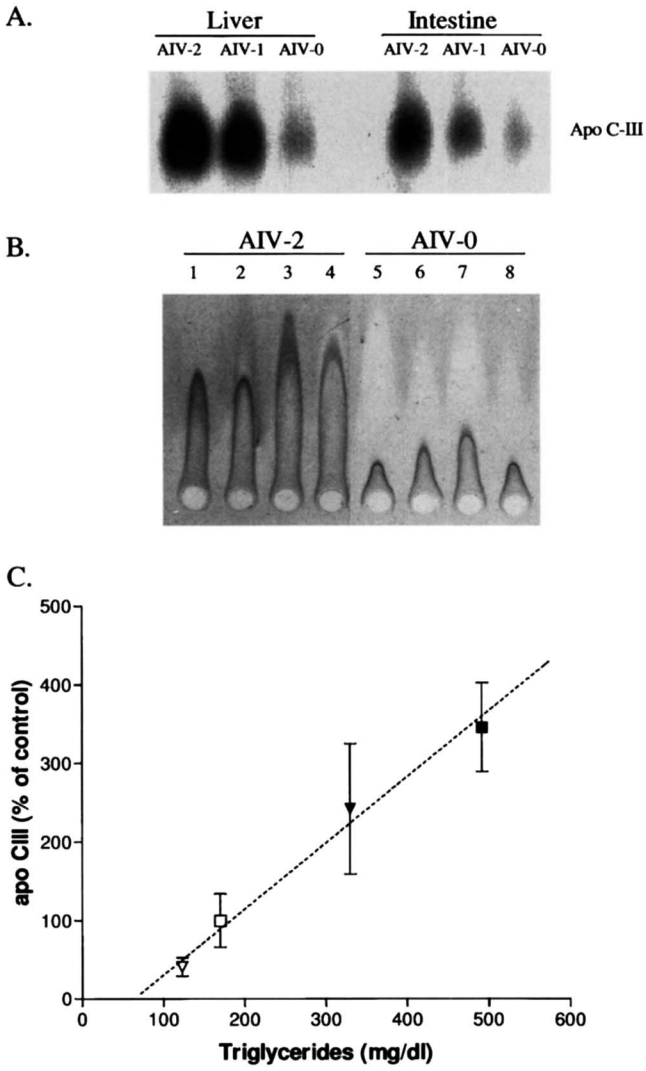


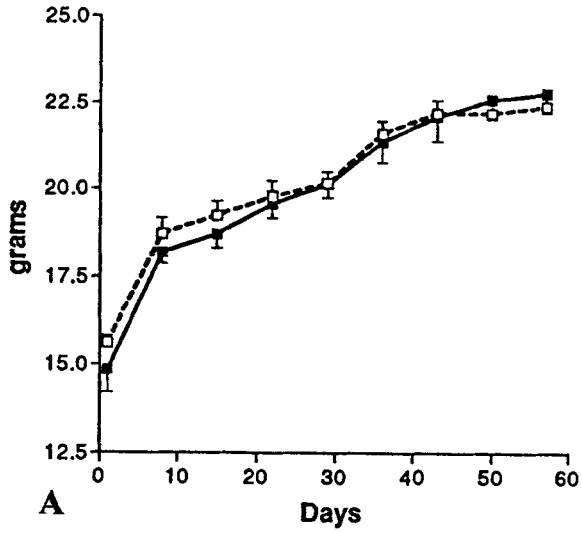
Fig. 4. ApoC-III deficiency in apoA-IV knockout mice. (A) Northern blot analysis of liver and intestinal apoC-III mRNA in control (AIV-2), heterozygous (AIV-1), and homozygous (AIV-0) apoA-IV knockout mice. Total RNA was hybridized with a mouse apoC-III specific riboprobe. (B) Quantification of apoC-III in mouse plasma by rocket immunoelectrophoresis. Typical analysis of 4–40 nl of control (lanes 1–4) and apoA-IV knockout mouse (lanes 5–8) plasma electrophoresed in the presence of cross-reactive goat anti-rat apoC-III antibody. All plasmas were normalized to the same standard mouse plasma which was always freshly thawed from frozen aliquots. (C) Transgenic complementation of apoC-III deficiency in apoA-IV knockout mice. Comparison of apoC-III and TG levels in control and knockout mice both with and without transgene-mediated apoC-III overexpression. Mice were generated by first crossing AIV-0 mice with apoC-III transgenic mice yielding progeny that were heterozygous at both alleles. These were then backcrossed to AIV-1 mice to produce littermates of all four genotypes. □, AIV-2 (n = 6); ∇, AIV-0 (n = 11); ■, AIV-2/C-III (n = 6); ▼, AIV-0/C-III (n = 10). Values represent means ± SD. The dotted line represents the trend line to which all points showed a strong correlation ($R^2 > 0.95$, $P < 0.001$).

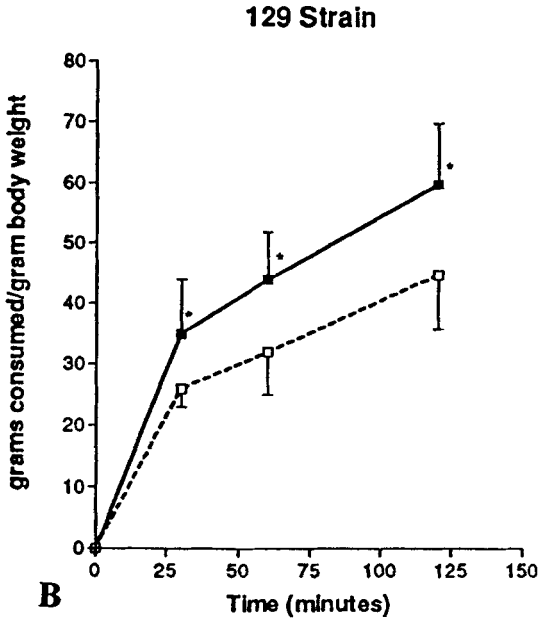
ance. Despite differences in baseline plasma triglyceride concentrations, triglycerides rose to similar levels in both the apoA-IV knockout and control mice (without Triton, Fig. 6B, with Triton, Fig. 6C). Finally, the ''single isotopic meal feed method'' was used to assess cholesterol absorption in apoA-IV knockout mice. As shown in **Table 4,** apoA-IV knockout mice had similar levels of cholesterol absorption as control mice. Thus many parameters of lipid absorption were normal in the apoA-IV knockout animals.

DISCUSSION

Previous studies using various in vitro and in vivo systems have not defined the function of apoA-IV, which is an abundant apolipoprotein. Therefore, in a further attempt to discover the true physiological role of this apolipoprotein, gene targeting in embryonic stem cells was used to knockout the apoA-IV gene and create apoA-IV-deficient mice. These mice had significant reductions in both cholesterol and triglyceride levels, which metabolic studies indicated were due to increased HDL cholesteryl ester FCR and decreased VLDL transport rate, respectively. Disruption of the apoA-IV gene caused decreased expression of the neighboring apoC-III gene, and this was shown to be responsible for the decreased triglyceride levels. The apoA-IV-deficient mice had normal cholesterol, triglyceride, and vitamin A absorption. They also had normal weight gain and ad lib food consumption. Male, but not female, apoA-IV-deficient mice showed increased food consumption after an 18-h fast.

The most significant lipoprotein abnormality in the apoA-IV knockout mouse was the decrease in plasma triglyceride, or VLDL levels. Metabolic studies indicated a decrease in VLDL transport rate while plasma free fatty acid levels, a major determinant of VLDL production, were normal. This suggested a role for apoA-IV in VLDL production. However, apoA-IV is not normally produced in the liver, implying that any effect must be indirect. Analysis of hepatic mRNA in the apoA-IV knockout mouse for the adjacent apoC-III gene revealed a 70% decrease in apoC-III mRNA. We have previously created human and mouse apoC-III transgenic mice that were hypertriglyceridemic (36, 41) and Maeda's group (42) had created apoC-III knockout mice that had decreased triglycerides. Thus decreased apoC-III production in the apoA-IV knockout mice was a plausible explanation for their decreased triglyceride levels. To confirm this and examine any contribution of apoA-IV to the hypotriglyceridemic phenotype, the apoA-IV knockout mice were crossbred to mouse apoC-





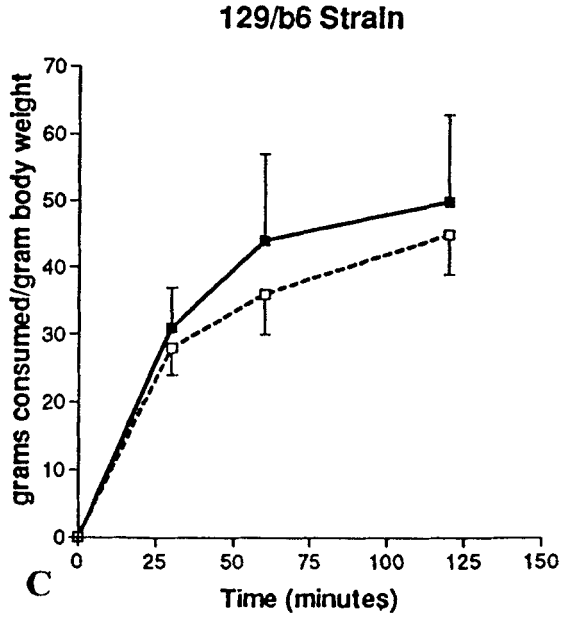


Fig. 5. Weight gain and feeding behavior in apoA-IV knockout mice. (A) Growth curve of female mice. Seven control (□) and four homozygous knockout (■) mice were maintained on chow diet and weighed once per week between 2 and 4 months of age. (B and C) Feeding behavior in outbred 129/bl6 and inbred 129 strain mice, respectively, after an 18-h fast. Feeding behavior was measured at 30, 60, and 120 min after replacing the food. $*P < 0.05$ (vs. control littermate). Data represents mean ± SD. Statistical analysis: Student's *t*-test.

III transgenic mice to create animals with varying amounts of apoC-III with and without apoA-IV. The results clearly showed that in the apoA-IV knockout mice the triglyceride abnormality was due to apoC-III deficiency independent of any apoA-IV contribution.

The metabolic basis of the hypotriglyceridemia in the apoA-IV knockout apoC-III-deficient mice appeared to be a decrease in hepatic VLDL production. This was surprising because we had previously shown in human and mouse apoC-III transgenic mice that the triglyceride elevation was primarily due to decreased VLDL FCR (36, 41), and Maeda et al. (42) had shown that hypotriglyceridemic apoC-III knockout mice had increased FCR of injected rat chylomicrons. Thus we expected that the apoA-IV knockout apoC-III-deficient mice would have increased VLDL FCR. However, our new findings were the opposite and suggest that decreased apoC-III expression can cause decreased VLDL production. ApoC-III is a constituent of VLDL and may be required for its normal packaging and secretion. Therefore, the fundamental metabolic mechanism of apoC-III deficiency may be different from apoC-III excess. It is not clear why apoC-III deficiency in the apoA-IV knockout mice caused decreased VLDL production, whereas the absence of apoC-III in the apoC-III knockout mice caused increased rat chylomicron FCR. It is possible that rat chylomicrons are not a valid tracer for endogenous VLDL. It is also possible, but unlikely, that apoC-III deficiency differs from apoC-III absence.

The other significant lipoprotein abnormality in the

TABLE 3. Growth and feeding behavior in apoA-IV knockout mice

	n	Body Weights		Food Intake	
		At Start of Experiment	Total Weight Gain	gms/day	gms/day/Average Weight[a]
Study I: 129/b6, 4 months old, 30 day observation					
AIV-2	7	25 ± 3	2.7 ± 1.3	4.9 ± 0.4	0.19 ± 0.02
AIV-0	6	26 ± 1	1.5 ± 1.1	5.4 ± 0.3[b]	0.20 ± 0.02
Study II: 129/b6, 5 months old, 21 day observation					
AIV-2	4	29 ± 1	2.3 ± 1.4	5.0 ± 0.5	0.17 ± 0.02
AIV-0	7	30 ± 2	2.2 ± 0.9	4.8 ± 0.4	0.16 ± 0.01
Study III: 129, 5 months old, 21 day observation					
AIV-2	6	25 ± 1	1.4 ± 0.8	4.2 ± 0.1	0.16 ± 0.01
AIV-0	8	24 ± 1	1.3 ± 0.9	4.2 ± 0.4	0.17 ± 0.02

Male mice were analyzed. Data represent means ± SD.

[a]Average weight over the experimental period was calculated as the sum of initial body weight plus one half of the total weight gain.

[b]$P < 0.05$ versus AIV-2.

apoA-IV knockout mouse was a decrease in HDL-C levels due to increased HDL cholesteryl ester FCR. In light of normal apoA-I levels in apoA-IV knockout mice as well as inconsistent. HDL-C changes in mice overexpressing (26) and deficient (42) in apoC-III, this aspect of the phenotype is most likely to be due to alterations in apoA-IV. To promote cholesterol efflux from peripheral cells and thus participate in reverse cholesterol transport, apoA-IV has been shown to form nascent HDL disks by combining with cellular lipids (43) as well as bind to cell-surface receptor sites (44). Findings in knockout mice lend support to a role for apoA-IV in reverse cholesterol transport as metabolic studies demonstrated HDL which is less stable and therefore more readily cleared in mice lacking apoA-IV.

On the otherwise wild-type background, apoA-IV deficiency was not associated with any significant change in intestinal lipid absorption. This was somewhat unexpected as apoA-IV is produced mainly in intestine (12), comprises 40% of the secreted protein of human intestine in organ culture (45), and fat feeding regulates apoA-IV expression levels (13–15). Previous studies with human apoA-IV transgenic mice, which expressed very high levels of the apolipoprotein, also failed to reveal a role for this apolipoprotein in intestinal lipid absorption (26). These studies taken together suggest that apoA-IV does not play a unique role in intestinal lipid absorption. Preliminary studies in double apoAIV/E knockout mice, aimed at elucidating a potential role for apoA-IV in atherogenesis (46) have revealed a marked reduction in intestinally derived lipoprotein remnants normally present in the plasma of apoE knockout mice (47). These findings suggest either complementary roles for apoA-IV and apoE in the metabolism of these lipoproteins or that an effect of apoA-IV on lipid absorption is masked in the otherwise wild type mouse by the extremely fast turnover of apoE-containing VLDL and that a marked increase in apoE-deficient β-VLDL half-life might serve to resolve an otherwise subtle effect. Ongoing studies will serve to better elucidate this issue and shed further light on the absorptive influence of apoA-IV.

Although apoA-IV deficiency was not associated with altered weight gain or routine food consumption, male, but not female, mice had increased food consumption after an 18-h fast. Fujimoto et al. (23, 24) have observed decreased feeding in rats after peripheral and central administration of rat apoA-IV, and have suggested that apoA-IV functions as a satiety factor. They have suggested that acute changes in apoA-IV levels may alter feeding behavior. If they are correct, then the chronically elevated or depressed apoA-IV levels in the transgenic and the knockout mice, respectively, may preclude detection of an apoA-IV effect on satiety. After a prolonged fast, things may be more similar to the acute infusion studies. Our finding of increased food intake after an 18-h fast in the apoA-IV-deficient mice is in the direction of what one would predict and therefore lends new support to Fujimoto's studies.

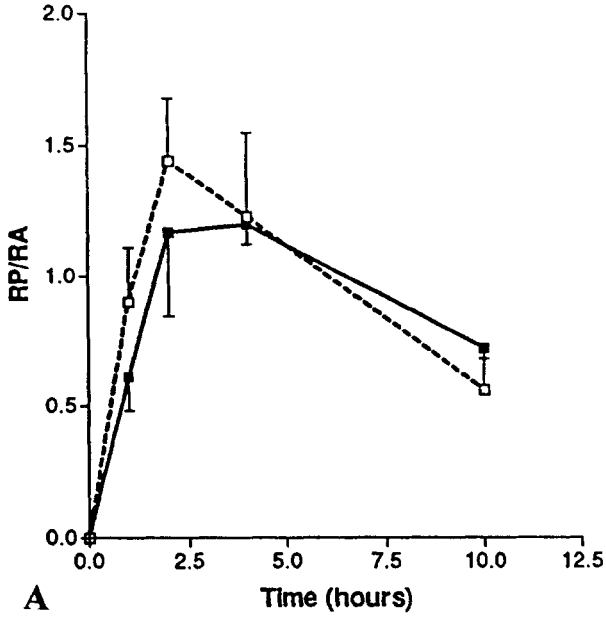
Our study of the apoA-IV knockout mouse reveals one potential limitation of the gene targeting technique, namely that one cannot assume that all aspects of the phenotype in the knockout mouse are due to the absence of the gene product under study. Fortunately, in this case we were able to breed in an independently

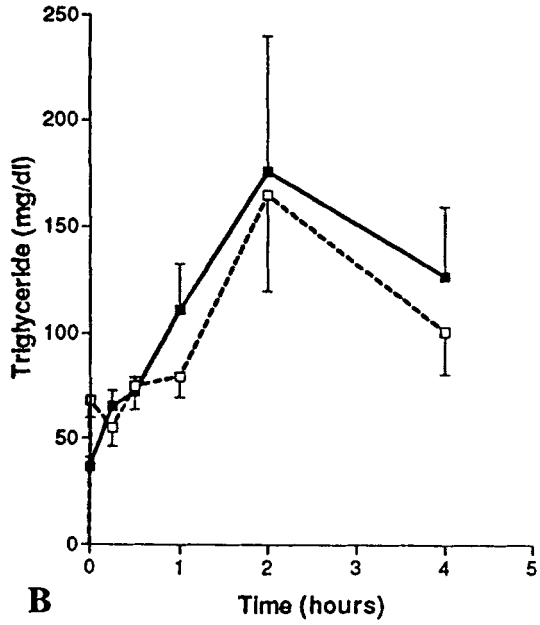
TABLE 4. Cholesterol absorption in apoA-IV knockout mice

	n	Weight	TC	Absorption
		g	*mg/dl*	%
AIV-2	5	21 ± 3	115 ± 31	50 ± 19
AIV-0	5	20 ± 2	74 ± 15[a]	57 ± 16

Cholesterol absorption in 3-month-old male mice was measured by the "single isotopic meal feeding" method. Data represent means ± SD.

[a]$P < 0.03$ versus AIV-2.





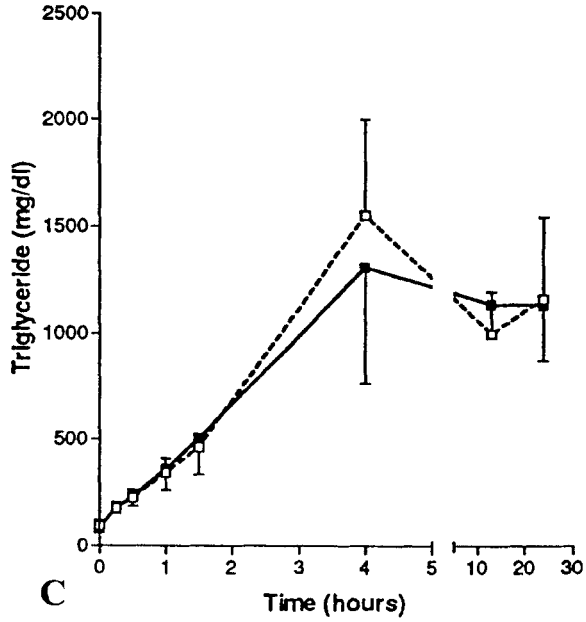


Fig. 6. Intestinal lipid absorption in apoA-IV knockout mice. (A) Vitamin A fat tolerance test. Intestinal absorption of retinyl palmitate was assessed in six male control (□) and five homozygous knockout (■) mice as described in Methods. At intervals up to 10 h, mice were bled, and serum retinyl palmitate (RP) content relative to retinyl acetate (RA, internal standard) was determined. (B) Corn oil fat loading test in the absence Triton WR 1339. Fasted female control (n = 5) and knockout (n = 6) mice were administered corn oil gastrically and plasma triglycerides were measured up to 4 h after injection. (C) Corn oil fat loading in the presence of i.v. Triton WR1339. Fasted female control (n = 7) and knockout (n = 7) mice were injected with Triton WR 1339 15 min prior to oral administration of 500 μl corn oil. Plasma triglycerides were measured up to 24 h after dosing. Data represent mean ± SD.

segregating transgene expressing the gene adjacent to apoA-IV, and show that one aspect of the knockout phenotype was due to decreased expression of the neighboring apoC-III gene. Maeda's group (42) described decreased expression of the apoA-IV gene in apoC-III knockout mice. Thus conservative disruption of the locus at either the apoA-IV or apoC-III genes by replacement of DNA with an equivalent amount of *neo* gene perturbs the expression of the adjacent gene. Olsen et al. (48) has recently reviewed three different lines of myogenic basic helix-loop-helix (*MRF4*) null mutants, arguing that the variability among phenotypes might be due to inadvertent alterations in the neighboring gene *MRF5*. The apoA-IV and apoC-III genes are separated by only 7 kb of intragenic sequence, which has been shown to contain an intestinal control region for the entire apoA-I/C-III/A-IV locus (49). It is possible that other regulatory elements in the locus may be affected by the replacement vectors used in targeting the apoA-IV and apoC-III genes.

Studies in apoA-IV knockout mice have highlighted issues with regard to gene targeting as well as uncovered

some of the functions of this apolipoprotein. The effect of gene knockout on neighboring genes was shown to be particularly important in the case of the apoA-IV/C-III/A-I gene complex and through complementation we have successfully assigned appropriate genes to particular aspects of the knockout phenotype. From these studies we may conclude that, independent of apoA-I, apoA-IV serves to increase HDL by extending its half-life in plasma. The mechanism by which the apolipoprotein achieves this effect remains unclear and is the subject of ongoing experiments. Yet, given the relatively high levels of circulating apoA-IV, the modest results of its complete absence seem less than satisfying, suggesting that apoA-IV serves in a "back-up" role that can be assumed by other gene products.

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