Intestinal Absorption of Dietary Cholesteryl Ester Is Decreased but Retinyl Ester Absorption Is Normal in Carboxyl Ester Lipase Knockout Mice[†]

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ABSTRACT: Carboxyl ester lipase (CEL; EC 3.1.1.13) hydrolyzes cholesteryl esters and retinyl esters in vitro. In vivo, pancreatic CEL is thought to liberate cholesterol and retinol from their esters prior to absorption in the intestine. CEL is also a major lipase in the breast milk of many mammals, including humans and mice, and is thought to participate in the processing of triglycerides to provide energy for growth and development while the pancreas of the neonate matures. Other suggested roles for CEL include the direct facilitation of the intestinal absorption of free cholesterol and the modification of plasma lipoproteins. Mice with different CEL genotypes [wild type (WT), knockout (CELKO), heterozygote] were generated to study the functions of CEL in a physiological system. Mice grew and developed normally, independent of the CEL genotype of the pup or nursing mother. Consistent with this was the normal absorption of triglyceride in CELKO mice. The absorption of free cholesterol was also not significantly different between CELKO (87 ± 26%, mean ± SD) and WT littermates (76 ± 10%). Compared to WT mice, however, CELKO mice absorbed only about 50% of the cholesterol provided as cholesteryl ester (CE). There was no evidence for the direct intestinal uptake of CE or for intestinal bacterial enzymes that hydrolyze it, suggesting that another enzyme besides CEL can hydrolyze dietary CE in mice. Surprisingly, CELKO and WT mice absorbed similar amounts of retinol provided as retinyl ester (RE). RE hydrolysis, however, was required for absorption, implying that CEL was not the responsible enzyme. The changes in plasma lipid and lipoprotein levels to diets with increasing lipid content were similar in mice of all three CEL genotypes. Overall, the data indicate that in the mouse, other enzymes besides CEL participate in the hydrolysis of dietary cholesteryl esters, retinyl esters, and triglycerides.

Carboxyl ester lipase (CEL;[1] EC 3.1.1.13), also called bile salt-stimulated lipase or cholesteryl ester hydrolase, is a multiple function lipolytic enzyme (*1*). In vitro, it hydrolyzes cholesteryl ester (CE), retinyl ester (RE), triglycerides, and lysophospholipids, with the activity against the first three substrates maximal in the presence of millimolar concentrations of trihydroxy bile salts. In many mammals, including humans, it is a major product of the pancreas and is also found in breast milk (*2*), blood plasma (*3*−*6*), liver (*7*−*9*), and aorta (*6*, *10*, *11*).

The primary role of CEL secreted by the pancreas has been thought to be the liberation of cholesterol (i.e., nonesterified or "free cholesterol") and retinol from their esters prior to their absorption in the intestine. In the developing neonate, breast milk CEL is also thought to aid in the digestion of triglycerides, and thereby provide energy for early growth and development while the neonatal pancreas matures and becomes able to secrete significant amounts of lipases (*12*, *13*).

CEL may have more diverse functions in lipid metabolism besides the digestive processing of lipid esters. For example, recent results from in vitro and in vivo studies have suggested that CEL in the intestinal lumen may directly facilitate the absorption of cholesterol in the free as well as the esterified form (*14*, *15*). In addition, CEL in the plasma may modify normal and modified lipoproteins and influence LDL cholesterol levels (*6*, *16*).

In studies of CEL function in vitro, contradictory results have often been obtained, perhaps due to the inability to faithfully reproduce the conditions present in vivo. Even in

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[1] Abbreviations: CE, cholesteryl ester; CEL, carboxyl ester lipase; CELKO, CEL knockout; FPLC, fast protein liquid chromatography; HDL-C, HDL cholesterol; lysoPC, lysophosphatidylcholine; PC, phosphatidylcholine; RE, retinyl ester; WT, wild type.

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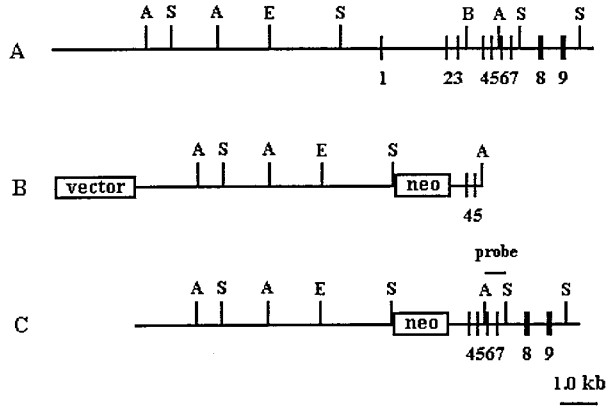

FIGURE 1: Strategy to disrupt the mouse CEL gene. (A) The mouse CEL locus. Exons are indicated by the black marks with numbers underneath. (B) The gene targeting construct with neomycin resistance cassette (neo) replacing the promoter and exons 1−3. (C) The mouse CEL locus after homologous recombination. A, *Avr*II; B, *Bam*HI, S, *Sac*I.

areas where there has been consistent agreement among different studies, such as the repeated demonstration of retinyl ester hydrolysis in vitro by CEL (*17*−*20*), there is little or no information establishing the existence or importance of the assumed function in the intact animal. To overcome these limitations, we have inactivated the mouse CEL gene by homologous recombination and have bred a colony of mice containing all three CEL genotypes [wild type (WT), heterozygote, knockout (CELKO)]. In this report, we have compared mice with these different gene dosages of CEL in regard to growth/development, intestinal lipid absorption, and plasma lipoprotein metabolism. A major result is that CEL is not required for the hydrolysis of dietary RE in the mouse. This novel finding implies the existence of at least one other enzyme that can hydrolyze RE, and additional evidence for such an enzyme in the mouse pancreas will be presented in the following paper (*21*) as part of extensive studies on the cellular uptake and processing of RE.

METHODS

Construction of Targeting Vector and Generation of CELKO Mice. The mouse CEL gene was cloned from a BALB/c lambda phage genomic library (Stratagene) using a PCR-amplified probe containing a rat CEL DNA sequence that included exons 2−4. Two overlapping clones were obtained, which contained 22 kb of mouse DNA extending from 9 kb 5′ to 4 kb 3′ of the CEL gene. Part of the wild-type allele is shown in Figure 1, row A. The targeting vector was constructed by inserting a 1.2 kb *Avr*II−*Bam*HI mouse CEL DNA fragment (which included exon 4) and an 8 kb *Sac*I−*Eco*RI fragment located 5′ to the gene into vector pPNT (a gift from Dr. Mario Capecchi, University of Utah) (Figure 1, row B). This strategy effectively replaces the promoter region and exons 1−3 with an intact neo gene (Figure 1, row C).

Ten micrograms of targeting vector was linearized by *Not*I and then transfected by electroporation of J1 embryonic stem cells (*22*). After selection in G418, surviving colonies were expanded, and PCR analysis was performed to identify clones that had undergone homologous recombination. PCR was done using primer pairs CE8 and Neo1. Primer CE8 is located in exon 6 directly outside the short arm of the targeting vector and has the sequence 5′-CATACCACTCT-GACTGATGGCTCTCC-3′. Primer Neo1 is located in the 5′-promoter region of the neo gene cassette and has the sequence 5′-TGCGAGGCCAGAGGCCACTTGTGTAGC-3′. Homologous recombination was confirmed by Southern blotting analysis.

Four correctly targeted ES cell lines were microinjected into C57BL/6J and BALB/cJ host blastocysts. These produced 10 chimeric male mice, 5 of which gave germ line transmission of the disrupted CEL gene.

Genotyping of Mice by PCR. CEL genotype analysis was done by PCR analysis of tail-tip-derived DNA samples. Each PCR reaction (50 μL) contained 4 μL of genomic DNA, 200 nM each primer, 200 μM each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, and 1 unit of AmpliTaq polymerase (Perkin-Elmer). The reaction mixture was heated to 99 °C for 7 min before the polymerase was added, and then subjected to 35 cycles of amplification consisting of 30 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C. Samples were analyzed on a 1% agarose gel.

Two upstream and one downstream primers were synthesized to differentiate wild-type, heterozygous, and homozygous knockout mice. The upstream primer A (in exon 2), 5′-CTCAGTCTCTTGGGTGGTGACTCTG-3′, and the downstream primer B (in exon 4), 5′-GGCGATCTCCTCCCCGT-CATA-3′, amplify a 680-bp fragment of the wild-type CEL gene. The upstream primer C (in the neo gene), 5′-GCCTACCCGCTTCCATTGCTC-3′, and the downstream primer B amplify a 665-bp fragment of the disrupted CEL gene. A positive result (i.e., a band of the expected size) with only primer sets A and B was scored as a wild type (WT). A positive result with only primer sets C and B was scored as disruption of both CEL alleles (CELKO). Positive results from both sets of primers indicated a heterozygote.

Determination of CEL Activity. Pancreases were obtained from Nembutol-anesthetized mice and immediately frozen in liquid nitrogen. Tissue was homogenized in 10 mM sodium phosphate (pH 6.2) containing 0.1 M NaCl, 1 mM EDTA, 0.02% sodium azide, 1.5% glycerol, and 0.02% soybean trypsin inhibitor. Breast milk samples were expressed manually from lactating mice. Plasma samples were obtained from blood collected from anesthetized mice through the orbital sinus with heparin as the anticoagulant. In preliminary studies, delipidation of plasma samples had no significant effect on the measurement of CEL activity, so untreated samples were used in subsequent determinations.

The CEL activity in each sample was determined radiometrically, based on procedures previously described using cholesteryl ester as the substrate (*23*). Typically, the reaction mixture (0.2 mL final volume) contained 50 mM Tris maleate (pH 7.0), 10 mM (for pancreatic and milk samples) or 50 mM (for plasma samples) sodium cholate, and an appropriately diluted enzyme source. The reactions were initiated by addition of 10 μL of ethanol containing 2 nmol of cholesteryl [1-^{14}C]oleate (Amersham, 56 mCi/mmol) with a specific activity of 25 μCi/μmol and incubated at 37 °C for 30 min. The released [^{14}C]oleate was extracted as described previously (*4*) and quantified by scintillation counting. Results are expressed in units of 1 nmol fatty acid released h^{-1} (μg of protein)$^{-1}$ (for pancreatic and breast milk samples) or 1 nmol of fatty acid released h^{-1} (mL of plasma)$^{-1}$ (for plasma samples). Protein concentrations of

pancreatic and breast-milk samples were determined by the Bradford method (*24*).

Absorption Studies. Intestinal absorption of cholesterol, CE, cholesteryl ether, RE, retinyl ether, or triglyceride was determined as follows:

The percentage absorption of a test dose of cholesterol or CE was measured by the Zilversmit double-isotope method (*25*). Briefly, each animal received 5 μCi of [1α, 2α(N)-^{3}H]cholesterol (46 Ci/mmol, Amersham) intravenously and 5 μCi of [^{14}C]cholesterol or [^{14}C]cholesteryl oleate in skim milk as an intragastric bolus. The ratios of [^{14}C]- to [^{3}H]-cholesterol in plasma were measured at 24, 42, 52, 65, and 78 h after administration and were used to calculate the percentage of cholesterol or cholesteryl oleate absorbed. In additional CE absorption studies, half of the experimental mice were pretreated with antibiotics to eliminate intestinal bacteria (*26*). Bacitracin, neomycin sulfate, and streptomycin sulfate, each at approximately 10 mg per day per mouse, were administered in drinking water for 5 days before the absorption test. A lack of bacterial growth from fecal cultures of antibiotic-treated mice was confirmed at the end of the 5-day pretreatment by microbiological examinations. The mice were kept on antibiotic treatment during the absorption study.

For cholesteryl ether absorption studies, [1α, 2α(N)-^{3}H]-cholesteryl oleoyl ether (10 μCi) in skim milk was administered by gavage. The appearance of ^{3}H in plasma was measured by scintillation counting at 1, 3, and 7 h after gavaging.

For the RE absorption study, [^{3}H]retinyl palmitate (2 000 000 dpm in 100 μL of peanut oil with 100 000 dpm of [^{14}C]cholesterol) was given to each mouse as an intragastric bolus. The appearance of ^{3}H and ^{14}C in plasma was determined by scintillation counting at 1, 2, 4, and 10 h after gavaging. Similarly, [^{14}C]retinyl hexadecyl ether (250 000 dpm in 100 μL of peanut oil) was given to mice to determine its intestinal absorption. Both of these radiolabeled compounds were synthesized as described previously (*27*).

Triglyceride absorption was determined as described previously (*28*). Briefly, mice were fasted overnight and treated with Triton WR 1339 (500 mg/kg body weight i.v.) to block lipolysis. After 15 min, mice were given an intragastric bolus of glycerol [^{3}H]trioleate (50 μCi) in corn oil (100 μL). Blood samples were drawn 0.5, 1, 1.5, and 8 h later, and the radioactivity was quantified by scintillation counting.

Analysis of Plasma Lipids and Lipoproteins. Blood samples were obtained from mice fed the following sequence of diets: normal Purina mouse chow diet for 8 weeks; the Western-type (*22*) high-fat diet (TD 88137, Teklad, 21% final fat content/0.15% cholesterol; w/w) for 2 weeks; and, finally, the atherogenic diet (TD 90221, Teklad, Purina mouse chow 5015, 15.75% final fat content, containing 7.5% cocoa butter, 1.25% cholesterol, and 0.5% cholic acid) for 2 weeks. Plasma samples from fed mice were obtained in the morning and from fasted mice in the evening (after food had been withdrawn for 8 h).

Plasma total cholesterol and triglyceride were determined enzymatically using commercial kits (Boehringer Mannheim). HDL cholesterol (HDL-C) was isolated and quantified from plasma by the Sigma HDL-reagent kit. Non-HDL-C (VLDL-cholesterol and LDL-cholesterol) was calculated as the difference between total cholesterol and HDL-C.

Plasma concentrations of phosphatidylcholine (PC) and lysophosphatidylcholine (lysoPC) were determined by methods modified from Switzer and Eder (*6*, *29*). Briefly, plasma lipids were extracted with 21 volumes of chloroform/methanol (2:1, v/v) and washed with 4 volumes of distilled water as described by Folch et al. (*30*). The phospholipids were separated by thin-layer chromatography (TLC) on silica gel G (500 μM as Uniplate, Analtech) with two solvent systems used in the same dimension: chloroform/methanol/water, 65:35:6; and chloroform/acetone/methanol/acetic acid/water, 6:8:2:2:1. Palmitoyl-PC and lysoPC (Sigma) were applied to TLC plates as standards. After visualization with iodine vapor, PC and lysoPC bands were scraped from the plates and assayed for inorganic phosphorus content according to the method of Rouser (*31*). The ratio of lysoPC to PC was calculated for each sample.

Plasma lipoprotein total cholesterol profiles were analyzed by FPLC using two Superose 6 columns in series (*32*). Plasma samples (200 μL) pooled from mice in the same experimental group were analyzed. Lipoproteins were eluted at a constant flow rate of 0.3 mL/min with 0.15 M NaCl containing 1 mM EDTA. Total cholesterol in the eluted fractions was determined enzymatically.

Statistical Analysis. Unless otherwise indicated, data are expressed as mean values $\pm$ standard deviation (SD) and were analyzed by Student's *t* test for statistical comparison. *P* values ≤ 0.05 were considered significant.

RESULTS

General Characteristics of CELKO Mice. CELKO mice were generated by inactivating the CEL gene in mouse stem cells through homologous recombination. Crosses between heterozygote mice yielded progeny with the expected 1:2:1 ratio of CELKO:heterozygote:WT genotypes (43:86:45). Both CELKO and heterozygote mice grew normally and appeared healthy. Pancreatic, breast milk, and plasma CEL activities were undetectable in CELKO mice, indicating a complete disruption of the CEL genes. The CEL activities in the pancreas and breast milk of heterozygote mice were approximately half that of WT mice (data not shown). CEL activities in plasma were low, and no difference was found between WT (2.3 ± 2 [nmol of fatty acid h^{-1} (mL of plasma)$^{-1}$ mean $\pm$ SD, $n = 6$]) and heterozygote mice (2.3 ± 2, $n = 6$).

Because CEL has been thought to play an important role in the digestion of milk fat in newborns, thereby providing energy for early growth and development, the body weight gain was measured every other day after birth. The growth rate before weaning of mice born to CELKO parents was the same as that of mice born to heterozygote parents (whose litters contained pups of all three genotypes). The average body weights of mice after weaning and on the chow, Western-type, and atherogenic diets were the same among CELKO, heterozygote, and WT littermates (data not shown).

Cholesterol Absorption. The majority of dietary cholesterol is in its free (i.e., nonesterified) form. The role of CEL in free cholesterol absorption has been controversial, with some in vitro studies showing that CEL either facilitates it (e.g., *14*) or does not (e.g., *33*). We measured the intestinal

absorption of free cholesterol in five mice of each genotype by using the Zilversmit double-isotope method (Methods). Plasma isotope ratios (^{14}C:^{3}H) were determined at time points up to 78 h after the initial dosing. As previously reported by Howles et al. (*34*), there were no significant differences among CELKO, heterozygote, and WT mice in free cholesterol absorption (averaging ~84%). Independent of their CEL genotypes, male mice tended ($P = 0.06$) to absorb less free cholesterol (78 ± 12%, $n = 9$) than females (94 ± 20%, $n = 6$), which was also previously observed (*34*).

Since CEL hydrolyzes CE in vitro to cholesterol and fatty acids in the presence of millimolar concentrations of cholate, it has been assumed that CEL is responsible in vivo for liberating cholesterol from its ester form prior to absorption by the intestine. Intestinal absorption of cholesterol derived from CE was tested as above, except [^{14}C]cholesterol was replaced by [^{14}C]cholesteryl oleate. For convenience, we will refer to the absorption of cholesterol derived from CE as CE absorption.

The results showed that intestinal CE absorption in CELKO mice did occur (38 ± 3%, $n = 7$), but it was decreased significantly ($P < 0.0001$) compared with that of WT littermate controls (83 ± 7%, $n = 6$). To exclude the possibility that CE might be taken up directly by the intestinal epithelial cells without hydrolysis, the absorption of a nonhydrolyzable radiolabeled analogue, [^{3}H]cholesteryl oleoyl ether, was tested. There was no detectable absorption by CELKO or WT mice, indicating that in addition to CEL, another enzyme or other enzymes hydrolyzed a fraction of intestinal CE.

Sterol-metabolizing enzymes of bacterial origin in the intestine may contribute to the hydrolysis of dietary CE (*35*) and conceivably could have contributed to the absorption of CE-derived cholesterol. To test this hypothesis, groups of WT and CELKO mice were given antibiotics in drinking water before and during the CE absorption study to eliminate the intestinal microflora (Methods). The CE absorption from mice treated with antibiotics (CELKO: 39 ± 1%, $n = 8$; WT: 84 ± 11%, $n = 7$) was essentially the same as the results obtained from untreated mice with the corresponding genotypes (see above), suggesting that intestinal bacterial enzymes were not involved in intestinal processing of dietary CE.

Retinyl Ester Absorption. A major source of vitamin A in animals consuming mixed diets is RE. As for CE, experimental data indicate that RE must be hydrolyzed prior to intestinal absorption, and CEL has been thought to be responsible for this hydrolysis (e.g., *36*). To test this hypothesis, [^{3}H]retinyl palmitate was given to WT, heterozygote, and CELKO mice as an intragastric bolus with [^{14}C]-cholesterol as an internal control for absorption (Methods). The radioactivities (both ^{3}H and ^{14}C) were measured in the plasma at subsequent time points. Surprisingly, no significant differences were found in the appearance and disappearance of ^{3}H in the plasma among these groups of different genotypes (Figure 2). HPLC analysis confirmed that the plasma ^{3}H was in the form of RE (data not shown).

The appearance and disappearance of plasma ^{14}C followed the same pattern as ^{3}H. To exclude the possibility that RE might be taken up directly (i.e., without hydrolysis) by intestinal epithelial cells, the absorption of nonhydrolyzable

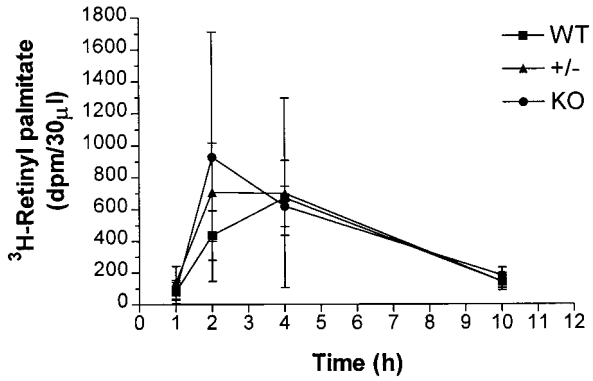

FIGURE 2: Effect of CEL expression on retinyl ester absorption. [^{3}H]retinyl palmitate (2 000 000 dpm) was administered as an intragastric bolus in 100 μL of peanut oil to five mice of each CEL genotype. At intervals up to 10 h blood samples were taken, and plasma radioactivity was determined as described under Methods. Data are displayed as mean ± SD. Abbreviations: WT (wild type); +/− (heterozygote); and KO (CEL knockout).

retinyl hexadecyl ether was tested in WT ($n = 4$, female) and CELKO ($n = 5$, female) mice. There was no appearance of ^{14}C in the plasma of any mouse of either genotype, indicating that retinyl hexadecyl ether was not directly absorbed. These data strongly suggest that there is another enzyme or other enzyme(s) responsible for hydrolyzing RE in the intestine of the mouse.

Triglyceride Absorption. Dietary lipids are 97% triglyceride, which are partially or completely hydrolyzed in the intestine before absorption. CEL can hydrolyze triglycerides in vitro and is thought to be required for their complete digestion in the neonatal period (*12*, *13*). To test whether the disruption of the CEL gene has any effect on intestinal triglyceride absorption, mice were treated by an intravenous injection of Triton WR 1339 [to block the lipolysis and clearance of plasma lipoproteins (*28*)] and then given an intragastric bolus of ^{3}H-labeled triolein in corn oil (Methods). The rate of appearance of ^{3}H in plasma was determined. No significant difference was found between WT and CELKO mice (Figure 3A). Interestingly, independent of their CEL genotypes, male mice tended to absorb less triglyceride than females (Figure 3B), as with the absorption of cholesterol or CE (see above).

Effects of Diets on Plasma Lipid and Lipoprotein Profiles. Groups of mice with different CEL genotypes were sequentially fed chow (8 weeks), Western (2 weeks), and atherogenic (2 weeks) diets (Methods). The FPLC−lipoprotein cholesterol profiles were determined in the fed and fasted states at the end of each of the three dietary periods. After a given diet, the FPLC profiles were similar between the plasma samples obtained from WT and CELKO mice (data not shown). The plasma samples were also used to quantify the levels of cholesterol (total, HDL, non-HDL) and triglycerides in plasma samples obtained in the fasted and fed states from mice of each genotype. These results are presented in Table 1. As expected (*37*), both the Western and atherogenic diets increased plasma total cholesterol concentrations measured in mice of each genotype sampled in either the fasted or the fed state ($P < 0.01$ for any comparison to the corresponding chow value). In Western diet-fed mice, both HDL-C and non-HDL-C increased, compared to that of chow-fed mice ($P < 0.01$); in atherogenic

Table 1: Plasma Lipid and Lipoprotein Concentrations (mg/dL) of Mice on Different Diets[a]

			fed				fasted			
diet	genotype	n[b]	TC	HDL-C	non-HDL-C	TG	TC	HDL-C	non-HDL-C	TG
chow	+/+	5	115 ± 25	93 ± 13	35 ± 7	170 ± 58	120 ± 22	79 ± 18	41.0 ± 9	115 ± 18
	+/−	9	103 ± 15	72 ± 21	46 ± 15	151 ± 27	109 ± 15	73 ± 12	35 ± 3	106 ± 27
	−/−	7	103 ± 29	70 ± 21	33 ± 8	151 ± 29	117 ± 24	74 ± 16	37 ± 11	99 ± 21
Western	+/+	5	213 ± 58	128 ± 31	85 ± 27	157 ± 63	213 ± 58	129 ± 40	84 ± 20	84 ± 27
	+/−	9	240 ± 48	149 ± 33	91 ± 21	198 ± 69	227 ± 39	148 ± 33	79 ± 15	102 ± 21
	−/−	7	219 ± 53	133 ± 34	86 ± 21	195 ± 90	202 ± 34	130 ± 21	72 ± 19	97 ± 16
atherogenic	+/+	5	286 ± 45	83 ± 29	203 ± 29	138 ± 20	359 ± 65	90 ± 34	285 ± 125	127 ± 29
	+/−	9	294 ± 66	86 ± 27	208 ± 68	137 ± 42	317 ± 99	84 ± 24	234 ± 102	146 ± 45
	−/−	7	333 ± 74	77 ± 40	252 ± 63	130 ± 19	333 ± 50	80 ± 37	261 ± 48	110 ± 21

[a] Data are given as mean ± SD. TC (total cholesterol), HDL-C, and TG (triglyceride) were determined enzymatically by using commercial kits as described under Methods. Non-HDL-C (VLDL-cholesterol and LDL-cholesterol) was calculated as the difference between total cholesterol and HDL-C. [b] There were 2 male and 3 female mice for the +/+ (wild type) genotype, 5 male and 4 female mice for +/− (heterozygote), and 2 male and 5 female mice for −/− (knockout).

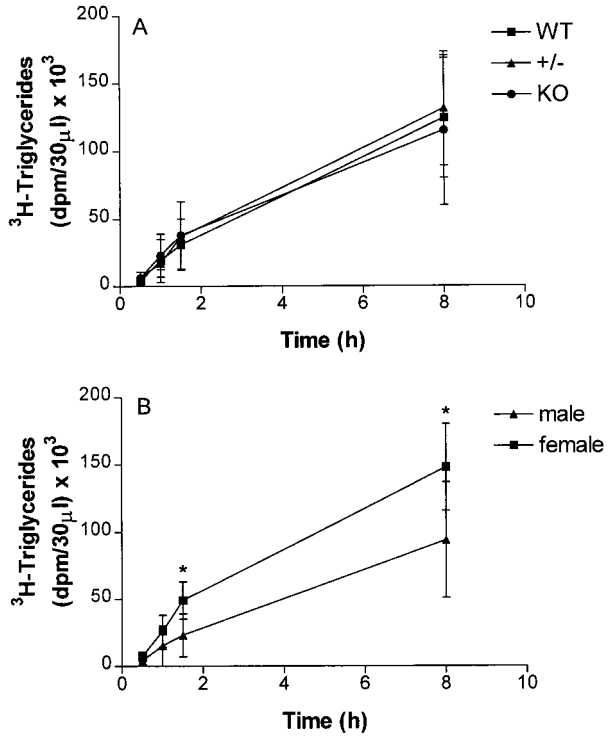

FIGURE 3: Effect of CEL expression and gender on triglyceride absorption. Panel A: Intestinal triglyceride absorption was assessed in 4 wild-type (WT), 2 heterozygote (+/−), and 6 CEL knockout (KO) mice as described under Methods. Mice were initially treated with Triton WR 1339 (to block lipolysis) and then given an intragastric bolus of glycerol [^{3}H]trioleate (50 μCi) in corn oil (100 μL). At intervals of 8 h, blood samples were taken, and plasma radioactivity was determined as described under Methods. Panel B: Comparison of triglyceride absorption in 6 male and 6 female mice independent of their CEL genotypes. Data are displayed as mean ± SD. Asterisk: differs at $P < 0.05$.

diet-fed mice, however, the increase in total cholesterol was largely due to the increase in non-HDL-C ($P < 0.005$).

Although mice have low CEL plasma activity, we tested whether lipoprotein composition varied among the genotypes. Ratios of free to total cholesterol in plasma were measured in chow-fed mice, and no significant differences were observed among the three genotypes (Table 2). Because lysoPC is an abundant plasma lipid species and CEL has lysophospholipase activity, ratios of lysoPC to PC in the plasma of chow-fed mice were also determined. There were comparable ratios among the three different CEL genotypes

Table 2: Plasma Ratios of Nonesterified Cholesterol (FC) to Total Cholesterol (TC)[a]

genotype	*n* (male + female)	FC/TC
+/+	5 (3 + 2)	0.19 ± 0.04
+/−	9 (3 + 6)	0.16 ± 0.03
−/−	4 (3 + 1)	0.18 ± 0.04

[a] Plasma samples were obtained from chow-fed mice of each genotype. Total cholesterol (TC; nonesterified + esterified) and nonesterified cholesterol (FC) were determined as described under Methods. Data are given as mean ± SD.

(WT, 0.46; heterozygote, 0.50; CELKO, 0.44; ratios determined in duplicate from plasma samples pooled from six mice of each CEL genotype).

DISCUSSION

In this report we have addressed the potential of CEL to influence a number of metabolic processes that fall into three major groups: the absorption of dietary lipids, neonatal growth and development, and plasma lipoprotein metabolism.

Role of CEL in the Absorption of Dietary Lipids. Since CEL exhibits hydrolytic activity in vitro for CE, RE, and triglyceride, it has been hypothesized that this enzyme plays important roles in the intestinal processing of these dietary components (*1*, *36*). The present studies have tested this hypothesis for each lipid by measuring in WT and CELKO mice the absorption of free and esterified cholesterol, RE, and triglyceride.

(A) Free and Esterified Cholesterol. Data from rat and human studies have suggested that pancreatic secretions contain a factor (or factors) that facilitates intestinal absorption of nonesterified cholesterol (*38*, *39*). CEL has been implicated as one such factor, although results from studies in vitro or in vivo have been contradictory (e.g., see *14*, *15*, *33*, *38*, *40*−*44*). Using CELKO and littermate heterozygote and WT mice, our results demonstrated that the absorption of cholesterol administered in the nonesterified, or free, form was indistinguishable among the genotypes. In contrast, compared to WT mice, in CELKO mice there was a 60% decrease in the absorption of cholesterol derived from CE.

These data are consistent with those of Howles et al. (*34*), and both sets of results raise the question of what regulates, in the absence of CEL, the absorption of a substantial fraction of CE-derived cholesterol. In the present studies, we have eliminated the possibilities of direct absorption of CE without

hydrolysis and hydrolysis mediated by bacterial enzymes. Overall, the data suggest that in mice CEL does not play a direct role in the absorption of nonesterified cholesterol, but it does hydrolyze a fraction of dietary CE, thereby releasing cholesterol that can be absorbed.

(B) Retinyl Ester Absorption. The role of CEL in the absorption of retinol provided by RE has been recently reviewed (*36*). It has been assumed that CEL is the only retinyl ester hydrolase secreted by the pancreas and that it has a critical role in the absorption of the approximately 50% of the dietary vitamin A in the typical American diet ingested in the form of RE. Thus, it was quite surprising that the absorption of retinol (derived from retinyl palmitate) was comparable between the CELKO and WT mice (Figure 2). Similar to the cholesteryl ether experiments, the lack of absorption of retinyl ether implied that hydrolysis was necessary for absorption and that it occurred in the absence of CEL activity. The simplest explanation for these results is that a non-CEL enzyme(s) catalyzed retinyl ester hydrolysis. Evidence for this in the CELKO mouse is given in the companion paper [van Bennekum et al. (*21*)].

Some potential non-CEL activities that may hydrolyze dietary RE in the intestine include the following: Emulsified retinyl palmitate can be hydrolyzed in vitro by pancreatic lipase, but its activity in vivo as a retinyl esterase would be severely inhibited by the millimolar concentrations of bile acids usually present in the intestine (*45*). Ong and colleagues (*20*) have found two kinds of retinyl esterase activities on the brush border membrane of rat small intestine. One was derived from the pancreas and preferentially hydrolyzed short-chain (i.e., not palmitate) retinyl esters in the presence of trihydroxy bile acids; it was presumed to be CEL (*20*). The other activity was intrinsic to the brush border membrane and preferentially hydrolyzed long-chain retinyl esters, and its activity was stimulated by trihydroxy and, unlike CEL, dihydroxy bile salts (*46*).

Whether the hydrolysis of RE and CE in the CELKO mice was accomplished by one of these candidates or other activities remains to be determined; the data in this report clearly support the existence of non-CEL enzyme(s) that hydrolyze(s) dietary RE in mice. At least one of these activities may be of pancreatic origin, as will be presented in the companion paper [van Bennekum et al. (*21*)].

(C) Triglyceride Absorption. Another in vitro hydrolytic activity of CEL is the bile salt-stimulated hydrolysis of triglyceride. It has been hypothesized that CEL can have a significant role in the processing in vivo of dietary triglyceride (*1*). In a number of mammalian species, including humans, the high content of CEL in breast milk is thought to supplement the relatively low level of triglyceride lipase activity in the secretions of the immature pancreas of the neonate (*12*, *13*). Nonetheless, there was no evidence for an important role of CEL in dietary triglyceride processing, as judged by the comparable absorption of triglyceride (Figure 3) and weight gain in the CELKO and WT mice. The latter finding indicates that there was no fat malabsorption in the neonatal period, despite a lack of CEL in either the breast milk of CELKO mothers or the pancreatic secretions of CELKO neonates.

Role of CEL in Infant Growth and Development. As noted above, CEL is expressed in the milk of many mammals and can serve as a digestive enzyme for lipid esters while the immature pancreas develops (*2*, *47*, *48*). A positive effect of CEL in neonatal growth and development has been proposed based on, for example, the addition of CEL to a formula diet for kittens leading to faster growth (*13*). Additionally, in human studies, increased CEL activity was associated with better fat absorption in infants (49), whereas decreased activity was found in the breast milk of undernourished nursing mothers and thought to compromise lipid digestion and the nutritional status of their infants (*48*).

Nonetheless, in agreement with Howles et al. (*34*), we found the growth rates of CELKO mice and their littermates (heterozygote and WT) to be similar, independent of the genotypes of either the pup or the nursing mothers. These data suggest, therefore, that CEL played, at most, a minor role in the growth and development of the neonatal mouse.

Role of CEL in the Metabolism of Plasma Lipoproteins. We (*6*) and others (*16*, *50*) have shown that CEL can circulate in the plasma of many mammals, including humans, and can modify in vitro the CE and lysoPC that are carried on normal and oxidized lipoproteins. Besides affecting the properties of lipoproteins by direct modifications, there is also the possibility that CEL, which like lipoprotein lipase has a heparin-binding site (*51*), could serve as a structural bridge between lipoproteins and cell-surface heparin sulfate proteoglycans and thereby affect the clearance and plasma levels of lipoproteins. Nonetheless, despite the use of different diets to alter plasma lipid and lipoprotein levels (Table 1), there were no differences in the results that were related to the CEL status of the mice. In addition, despite its activity as a cholesteryl ester hydrolase and lysophospholipase (*52*), the fraction of total cholesterol that was nonesterified and the plasma levels of lysoPC were comparable among the animal groups (Table 2 and Results). One important possibility to explain these results is that wild-type mice, in contrast to many other mammals, appear to have naturally low plasma levels of CEL activity (Results); compared to rat plasma (*4*), they are $<5\%$. Thus, further reduction of the plasma CEL activity in the KO mouse would not be not likely to result in perceptible consequences, which is what we observed. Overall, our data suggest that in the wild-type mouse, CEL has no significant effects on plasma lipoprotein levels or composition.

In summary, we have used gene-targeting techniques to develop mice to study the effects of CEL gene dosage on lipid and lipoprotein metabolism as well as on growth and development. CELKO mice, compared to either heterozygotes or WT littermate controls, had similar growth rates, plasma lipoprotein levels, plasma (lysoPC/PC and nonesterified/total cholesterol) ratios, and absorption of nonesterified cholesterol, RE, and triglyceride. The reduction of the absorption of cholesterol derived from CE suggests that CEL does function in vivo as a cholesteryl ester hydrolase in the intestine. That this reduction in absorption was partial, taken together with normal RE absorption in the CELKO mouse, however, implies the existence of additional digestive enzyme(s) that can function in roles previously attributed to CEL.

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REFERENCES

1. Wang, C. S., and Hartsuck, J. A. (1993) *Biochim. Biophys. Acta* **1166**, 1−19.
2. Blackberg, L., Lombardo, D., Hernell, O., Guy, O., and Olivecrona, T. (1981) *FEBS Lett. 136*, 284−288.
3. Goldberg, I. J., Blaner, W. S., and Goodman, D. S. (1986) *Arch. Biochem. Biophys. 24*, 580−584.
4. Harrison, E. H. (1988) *Biochim. Biophys. Acta 963*, 28−34.
5. Lombardo, D., Montalto, G., Roudani, S., Mas, E., Laugier, R., Sbarra, V., and Abouakil, N. (1993) *Pancreas 8*, 581−588.
6. Shamir, R., Johnson, W. J., Morlock-Fitzpatrick, K., Zolfaghari, R., Li, L., Mas, E., Lombardo, D., Morel, D. W., and Fisher, E.A. (1996) *J. Clin. Invest. 97*, 1696−1704.
7. Zolfaghari, R., Harrison, E. H., Ross, A. C., and Fisher, E. A. (1989) *Proc. Natl. Acad. U.S.A. 86*, 6913−6916.
8. Zolfaghari, R., Harrison, E. H., Han, J. H., Rutter, W. J., and Fisher, E. A. (1992) *Arterioscler. Thromb. 12*, 295−301.
9. Camulli, E. D., Linke, M. J., Brockman, H. L., and Hui, D. Y. (1989) *Biochim. Biophys. Acta 1005*, 177−182.
10. Kothari, H. V., Miller, B. F., and Kritchevsky, D. (1973) *Biochim. Biophys. Acta 296*, 446−454.
11. Kothari, H. V., and Kritchevsky, D. (1975) *Lipids 10*, 322−330.
12. Mehta, N. R., Jones, J. B., and Hamosh, M. (1982) *J. Pediatr. Gastroenterol. Nutr. 1*, 317−326.
13. Wang, C. S., Martindale, M. E., King, M. M., and Tang, J. (1989) *Am. J. Clin. Nutr. 49*, 457−463.
14. Lopez-Candales, A., Bosner, M. S., Spilburg, C. A., and Lange, L. G. (1993) *Biochemistry 32*, 12085−12089.
15. Lopez-Candales, A., Grosjlos, J., Sasser, T., Buddhiraju, C., Scherrer, D., Lange, L. G., and Kumar, V. B. (1996) *Biochem. Cell. Biol. 74*, 257−264.
16. Brodt-Eppley, J., White, P., Jenkins, S., and Hui, D. Y. (1995) *Biochim. Biophys. Acta 1272*, 69−72.
17. Lombardo, D., Fauvel, J., and Guy, O. (1980) *Biochim. Biophys. Acta 611*, 136−146.
18. Erlanson, C., and Borgstrom, B. (1968) *Biochim. Biophys. Acta 167*, 629−631.
19. Harrison, E. H., and Grad, M. Z. (1989) *J. Biol. Chem. 264*, 17142−17147.
20. Rigtrup, K. M., and Ong, D. E. (1992) *Biochemistry 31*, 2920−2926.
21. van Bennekum, A. M., Li, L., Piantedosi, R., Shamir, R., Vogel, S., Fisher, E. A., Blaner, W. S., and Harrison, E. H. (1999), *Biochemisry 38*, 4150−4156.
22. Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setala, K., Walsh, A., Verstuyft, J. G., Rubin, E. M., and Breslow, J. L. (1992) *Cell 71*, 343−353.
23. Harrison, E. H., Smith, J. E., and Goodman, D. S. (1979) *J. Lipid Res. 20*, 760−771.
24. Redinbaugh, M. G., and Campbell, W. H. (1985) *Anal. Biochem. 147*, 144−147.
25. Zilversmit, D. (1972) *Proc. Soc. Exp. Biol. Med. 140*, 862−865.
26. Kinouchi, T., Kataoka, K., Miyanishi, K., Akimoto, S., and Ohnishi, Y. (1993) *Carcinogenesis 14*, 869−874.
27. Blaner, W. S., Dixon, J. L., Moriwaki, H., Martino, R. A., Stein, O., Stein, Y., and Goodman, D. S. (1987) *Eur. J. Biochem. 164*, 973−981.
28. Aalto-Setälä, K., Bisgaier, C. L., Ho, A., Kieft, K. A., Traber, M. G., Hayden, H. J., Ramakrishnan, R., Walsh, A., Essenburg, A. D., and Breslow, J. L. (1994) *J. Clin. Invest. 93*, 1776−1786.
29. Switzer, S., and Eder, H. A. (1965) *J. Lipid Res. 6*, 506−511.
30. Folch, J., Lees, M., and Stanley, G. H. S. (1957) *J. Biol. Chem. 226*, 497−509.
31. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) *Lipids 5*, 494−496.
32. Kieft, K. A., Bocan, T. M. A., and Krause, B. R. (1991) *J. Lipid Res. 32*, 859−866.
33. Shamir, R., Johnson, W. J., Zolfaghari, R., Lee, H. S., and Fisher, E. A. (1995) *Biochemistry 34*, 6351−6358.
34. Howles, P. N., Carter, C. P., and Hui, D. Y. (1996) *J. Biol. Chem. 271*, 7196−7202.
35. Nonogak, K., Moser, A. H., Pan, X. M., Staprans, I., Grunfeld, C., and Feingold, K. R. (1995) *J. Lipid Res. 36*, 1987−1995.
36. Harrison, E. H. (1993) *Biochim. Biophys. Acta 1170*, 99−108.
37. Nishina, P. M., Verstuyft, J., and Paigen, B. (1990) *J. Lipid Res. 31*, 859−869.
38. Gallo, L. L., Clark, S. B., Myers, S., and Vahouny, G. V. (1984) *J. Lipid Res. 25*, 604−614.
39. Bell, C. C., Jr., and Swell, L. (1968) *Proc. Soc. Exp. Biol. Med. 128*, 575−577.
40. Vahouny, G. V., and Treadwell, C. R. (1958) *Am. J. Physiol. 195*, 516−520.
41. Watt, S. M., and Simmonds, W. J. (1981) *J. Lipid Res. 22*, 157−165.
42. Gallo, L. L., Newbill, T., Hyun, J., Vahouny, G. V., and Treadwell, C. R. (1977) *Proc. Soc. Exp. Biol. Med. 156*, 277−281.
43. Bhat, S. G., and Brockman, H. L. (1982) *Biochem. Biophys. Res. Commun. 109*, 486−492.
44. Huang, Y., and Hui, D. Y. (1990) *J. Lipid Res. 31*, 2029−2037.
45. Ong, D. E. (1993) *J. Nutr. 123*, 351−355.
46. Rigtrup, K. M., Kakkad, B., and Ong, D. E. (1994) *Biochemistry 33*, 2661−2666.
47. Hernell, O., and Blackberg, L. (1994) *J. Pediatr. 125*, S56−S61.
48. Dupuy, P., Sauniere, J. F., Vis, H. L., Leclaire, M., and Lombardo, D. (1991) *Lipids 26*, 134−138.
49. Alemi, B., Hamosh, M., Scanlon, J. W., Salzman-Mann, C., and Hamosh, P. (1981) *Pediatrics 68*, 484−489.
50. Bhakdi, S., Dorweiler, B., Kirchmann, R., Torzewski, J., Weise, E., Tranum-Jensen, J., Walev, I., and Wieland, E. (1995) *J. Exp. Med. 182*, 1959−1971.
51. Bosner, M. S., Gulick, T., Riley, D. J., Spilburg, C. A., and Lange, L. G. (1989) *J. Biol. Chem. 264*, 20261−20264.
52. Han, J. H., Stratowa, C., and Rutter, W. J. (1987) *Biochemistry 26*, 1617−1625.

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