Carboxyl Ester Lipase Overexpression in Rat Hepatoma Cells and CEL Deficiency in Mice Have No Impact on Hepatic Uptake or Metabolism of Chylomicron-Retinyl Ester†

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ABSTRACT: To study the role of carboxyl ester lipase (CEL) in hepatic retinoid (vitamin A) metabolism, we investigated uptake and hydrolysis of chylomicron (CM)-retinyl esters (RE) by rat hepatoma (McArdle-RH7777) cells stably transfected with a rat CEL cDNA. We also studied tissue uptake of CM-RE in CEL-deficient mice generated by targeted disruption of the CEL gene. CEL-transfected cells secreted active enzyme into the medium. However, both control and CEL-transfected cells accumulated exogenously added CM-RE or CM remnant (CMR)-derived RE in equal amounts. Serum clearance of intravenously injected CM-RE and cholesteryl ester were not different between wild-type and CEL-deficient mice. Also, the uptake of the two compounds by the liver and other tissues did not differ. These data indicate that the lack of CEL expression does not affect the uptake of dietary CM-RE by the liver or other tissues. Moreover, the percentage of retinol formed in the liver after CM-RE uptake, the levles of retinol and retinol-binding protein in serum, and retinoid levels in various tissues did not differ, indicating that CEL deficiency does not affect hepatic retinoid metabolism and retinoid distribution throughout the body. Surprisingly, in both pancreas and liver of wild-type, heterozygous, and homozygous CEL-deficient mice, the levels of bile salt-dependent retinyl ester hydrolase (REH) activity were similar. This indicates that in the mouse pancreas and liver an REH enzyme activity, active in the presence of bile salt and distinct from CEL, is present, compatible with the results from our accompanying paper that the intestinal processing and absorption of RE were unimpaired in CEL-deficient mice.

Carboxyl ester lipase (CEL)[1] (*1*)(EC 3.1.1.13) [also known as bile salt-activated lipase (*2*), bile salt-dependent cholesteryl ester hydrolase (CEH) (*3*), or bile salt-dependent retinyl ester hydrolase (REH) (*2*)] is capable of catalyzing the hydrolysis of a variety of neutral lipid ester substrates, including cholesteryl ester (CE), triglyceride (TG), and retinyl ester (RE), and acidic lipids, such as phospholipid (PL) and lyso-PL (*4, 5*). In in vitro assays with neutral lipid esters, the enzyme requires millimolar concentrations of trihydroxy bile salts, such as sodium cholate, for maximal activity (*3, 6*). The enzyme is synthesized in the mammalian pancreas and secreted into the intestinal lumen (*4*). CEL is also synthesized and secreted by mammary tissue (*7*), and the liver (*8*−*11*).

A role in vivo for pancreatic CEL was suggested by the recent demonstration that the intestinal absorption of esterified cholesterol, but not that of free cholesterol, was impaired in a CEL-deficient mouse strain, generated by targeted disruption of the CEL gene (*12*). In a cell culture system using human intestinal CaCo-2 cells, CEL also enhanced the uptake of CE-derived cholesterol, but not that of free cholesterol (*13*). The metabolic role of CEL in mammalian milk is also fairly well established; CEL appears to be involved in the digestion of a variety of lipids in the suckling newborn (*14, 15*).

The role of CEL in the liver is not defined. While it was initially thought that the activity detected in liver may be the consequence of uptake of the pancreatic enzyme (*6*), it is now clear that the enzyme is expressed in, and secreted by, the liver (*3, 8*−*10*). The secreted enzyme may act in the space of Disse where it may be involved in the metabolism of neutral lipid esters (CE, TG, RE) of chylomicrons (CM) or chylomicron remnants (CMR). In vitro studies have shown that CEL is a potent bile salt-dependent retinyl ester hydrolase (REH) (*16, 17*); however, the in vivo significance of this activity remains unclear. Little is known about the

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[1] Abbreviations: CE, cholesteryl ester; CEH, cholesteryl ester hydrolase; CEL, carboxyl ester lipase; CELKO, CEL-deficient; CM, chylomicron; CMR, chylomicron remnant; DMEM, Dulbecco's modified Eagle's medium; HL, hepatic lipase; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; PL, phospholipid; RE, retinyl ester; REH, retinyl ester hydrolase; RBP, retinol-binding protein; SD, standard deviation; TG, triglyceride; WT, wild type.

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relative contribution that CEL plays in the uptake and initial hydrolysis of CMR-RE in vivo. In the accompanying paper by Weng et al. (*18*), we showed that the intestinal absorption of [^{3}H]retinyl palmitate in mice was not affected by the lack of CEL expression. In this study, we explored directly the effect of CEL deficiency on the hepatic uptake of CM-derived [^{3}H]-RE (and [^{14}C]-CE). We also studied the effect of CEL expression on the uptake and hydrolysis of CM- or CMR-RE in McArdle-RH7777 rat hepatoma cells which were stably transfected with CEL cDNA.

Our results indicate that CEL deficiency had no effect on hepatic uptake of CM-derived RE. Moreover, the expression of CEL in the hepatoma cells had no effect on the uptake or hydrolysis of CM- or CMR-RE. The absence of CEL did not influence serum levels of retinol and retinol-binding protein (RBP), or tissue total retinol levels. Finally, our studies demonstrated the existence of a novel REH activity in the mouse pancreas which is distinct from CEL, consistent with our previous results demonstrating normal RE absorption in CEL-deficient mice (*18*).

MATERIALS AND METHODS

Production of CEL-Deficient Mice. C57BL/6J mice lacking CEL (CELKO mice) were generated using standard procedures for targeted gene disruption (*18*). The genotypes of CEL expressing wild-type (WT), homozygous-deficient (CELKO), or heterozygous mice were determined using a polymerase chain reaction procedure, as described (*18*).

Production and Culture of McA-RH7777 Cells Stably Overexpressing Rat Pancreatic CEL Protein. McA-RH7777 rat hepatoma cells, obtained from ATCC, were either transfected with control plasmid (pMAM neo, which expresses resistance to G418) or cotransfected with an SV40-expression plasmid containing rat pancreatic CEL cDNA and pMAM neo, according to procedures described previously (*19*). Briefly, after the transfection by the $CaPO_4$ procedure, cells were grown in medium containing 400 μg/mL G418 for 3 weeks. Surviving control and CEL positive clones were pooled and grown as described below. Control and CEL-transfected McA-RH7777 cells were maintained at 37 °C in a humidified CO_2 atmosphere (95% air, 5% CO_2) in 25 or 75 cm^2 Primaria flasks (Falcon, Franklin Lakes, NJ) in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker, Walkersville, MD) containing 20% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO) and G418 (final concentration 200 IU/mL; Gibco BRL, Gaithersburg, MD).

Preparation and Characterization of Radiolabeled Chylomicrons (CM) and Chylomicron Remnants (CMR). For preparation of rat CM containing [^{3}H]-RE, chyle containing [^{3}H]-RE was isolated from the cannulated mesenteric lymph duct of male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), according to a procedure described previously (*20*). Briefly, four rats were fasted overnight and were each given 0.2 mL of a 1:1 solution of olive oil and corn oil containing 4 mg of α-tocopherol (Supelco, Bellefonte, PA), 4 mg of retinol (Sigma) and 200 μCi of [^{3}H]retinol (retinol-[11,12-^{3}H], 35.2 Ci/mmol; ARC Inc., St. Louis, MO). The chyle which was collected from the rats for periods up to 24 h was pooled, overlayered with 0.05% ethylenediaminetetraacetic acid in saline, pH 7.4, and centrifuged at 39 000 rpm for 25 min at 18 °C to isolate CM which were stored at 4 °C in the dark for periods up to 4 days prior to use. For generation of CM containing [^{3}H]-RE plus [^{14}C]-CE, a similar protocol was used except that, in addition to [^{3}H]-retinol, 40 μCi of [^{14}C]cholesterol (cholesterol-[4-^{14}C], 51 mCi/mmol; Dupont NEN, Boston, MA) was given to each animal.

Rat CMR containing [^{3}H]-RE were prepared according to Lenich and Ross (*21*). Briefly, postheparin plasma was obtained from a fasted male Sprague Dawley rat which had been injected with 500 IU of heparin/kg body weight 10 min earlier. Then 117 mg of CM-TG, 3.97 g of bovine serum albumin, 3.5 mL of postheparin plasma, and 0.2 M Trizma buffer, pH 8, in a final volume of 70 mL were incubated in the dark at 37 °C until the turbidity of the mixture had cleared (4 h). CMR were reisolated from the incubation mixture according to Gustafson et al. (*22*).

After isolation, for both CM and CMR containing [^{3}H]-RE, less than 5% of total CM-retinoid was present as free retinol, as confirmed by high-performance liquid chromatography (HPLC) analysis (see below). However, for CM also containing [^{14}C]-CE, 35% of the total cholesterol was present as free cholesterol, as assessed by thin-layer chromatography performed according to published procedures (*13*). This value is in agreement with previous studies where CM containing radiolabeled CE are generated in vivo (*23*). The purified CM- and CMR-[^{3}H]-RE consisted of 65% retinyl palmitate, the remainder being other acyl esters.

Small aliquots of CM and CMR preparations were extracted according to Bligh and Dyer (*24*). The extracts were evaporated and redissolved in a small aliquot of acetonitrile/isopropyl alcohol (1:1 v/v), which was used directly for TG analysis using a commercially available TG kit (GPO Trinder, Sigma). The morphology of the CM and CMR preparations, as well as the size distribution of the particles, was assessed by electron microscopy employing a negative staining procedure (*25*). The size distribution of the CM preparation ranged from 250 to 3750 nm [averaging 1108 ± 318 nm (SD)], and that of the CMR preparation ranged from 50 to 2500 nm (averaging 633 ± 639 nm).

Cell Uptake Experiments. Cell culture conditions for the CM- or CMR-[^{3}H]-RE uptake experiments were essentially the same as described previously (*3, 19*). For a given experiment, both control and CEL-transfected cells were plated at a density of 1.5×10^6 cells per well into 6-well Primaria plates (Falcon) and were allowed to grow for 24 h. Each well contained 2 mL of DMEM containing 20% fetal bovine serum and G418 as described above. This medium was removed and changed to DMEM containing 20 μM bovine serum albumin for 18 h in order to allow the accumulation of CEL-protein in the medium of the CEL-transfected cells. Either 0.1 mL of H_2O or 0.1 mL of 0.2 mM sodium cholate (final concentration: 10 μM) was added to each well, and immediately thereafter an appropriately diluted CM or CMR preparation containing [^{3}H]-RE was added in a volume of 0.1 mL.

Cells were incubated at 37 °C for various periods of time, and incubations were stopped by placing the cells on ice, removing the medium by transferring it to an ice-cold test tube, and adding 2 mL of ice-cold PBS to each well. Cells were then scraped off the plate and centrifuged for 10 min at 1500 rpm at 4 °C. The cells were washed 2 more times by resuspension in PBS and centrifugation. After the third

wash, cells were resuspended in 0.5 mL of ice-cold PBS and kept on ice, for periods not exceeding 1 h, until further processing. The complete cell suspension was used for retinoid extraction. Aliquots (200 μL) of the collected media were diluted to 500 μL with PBS prior to processing as described below.

Extraction and Quantitation of Retinoids from Cells and Media. Retinoids were extracted according to published procedures (*26*). Briefly, to both cells and medium were added 4 volumes (2 mL) of absolute ethanol containing the internal standard retinyl acetate (Sigma), and retinoids were partitioned into 2 volumes (5 mL) of hexane. After evaporation of hexane under a gentle stream of N_2, the sample was redissolved in absolute ethanol for injection onto the HPLC. Retinol and RE were separated by reverse-phase HPLC essentially according to Harrison et al. (*26*). The mobile phase was 100% methanol delivered at a flow rate of 1.5 mL/min. Retinoids were detected by monitoring the absorbance at 326 nm. A small aliquot of the extract was kept for liquid scintillation counting. The remainder of the extract was left to dry, after which protein levels were determined (see below).

Experiments in WT, Heterozygous, and CELKO Mice. For studies on the uptake of [^{3}H]-RE and [^{14}C]-CE from rat CM, 5 WT, 12 heterozygous, and 3 CELKO mice (weighing approximately 25 g, and all obtained from the same heterozygous breeding pair) were fasted overnight. The next day, animals were anesthesized with an intraperitoneal injection of a mixture of ketaset (100 mg/kg body weight) and zylazin (20 mg/kg body weight). Then 100 μL of rat CM containing 5.4 mg of TG and a trace amount of [^{3}H]-RE (30 nmol, 0.5 μCi) and [^{14}C]-CE (2.4 nmol, 0.12 μCi) was injected into the right jugular vein of each mouse. Blood samples were obtained from a lateral tail vein at 5, 10, and 30 min. At 30 min, animals were bled, and total body perfusion was performed using an ice-cold solution of 0.9% NaCl containing 0.5 mM ethylenediaminetetraacetic acid at a rate of 4.5 mL/min for 2−3 min in order to remove residual blood from the tissues. Liver, spleen, kidney, lung, heart, perirenal fat, gastrocnemius muscle, and brain were excised and immediately frozen in liquid N_2 for storage at −70 °C. Blood samples were allowed to clot at 4 °C and centrifuged at 2000*g* for 15 min (4 °C) to separate the serum from the clot.

Liquid Scintillation Counting of Mouse Tissue Extracts. Tissues were processed and extracted using chloroform/methanol (2:1 v/v) as described previously (*27*). The extracts were evaporated and redissolved in 20 mL of Scintiverse liquid scintillation counting solution (Fisher Scientific, Pittsburgh, PA). ^{3}H and ^{14}C were assayed simultaneously in a Beckman LS 3801 scintillation counter (Beckman Instruments, Fullerton, CA) using a sensitive quench−correction program. Spillover of ^{14}C counts into the ^{3}H channel, and vice versa, amounted to 7.4 and 0.7%, respectively. For calculations of total tissue weight, a value of 2.75% of total body weight was used for estimating total serum volume (*28*). Total weight of gastrocnemius muscle was estimated to be 4% (*28*).

In livers from mice that had been injected with CM-[^{3}H]-RE, the percentage of [^{3}H]-retinol formed at 30 min was determined by analysis of a portion of the chloroform extract. Extracts were evaporated under N_2, redissolved in hexane, and applied to 10% deactivated alumina columns in order to separate retinol and RE as described by Lenich and Ross (*21*).

HPLC Procedure for Determination of Retinoid Content in Various Tissues of WT, Heterozygous, and CELKO Mice. Tissues obtained from mice were homogenized in 2 mL of PBS, after which an equal volume of absolute ethanol containing the internal standard retinyl acetate was added. Retinol and RE were extracted in 5 mL of hexane, which was subsequently evaporated under a gentle stream of N_2. The extract was redissolved in a small aliquot of benzene for injection onto the HPLC. Retinol and RE (retinyl linoleate, retinyl oleate, retinyl palmitate, and retinyl stearate) were analyzed using a reverse-phase HPLC procedure as described (*29*) using a 4.6 × 250 mm 5 μm Beckmann Ultrasphere C18 column (Beckmann Instruments, Inc.). Retinoids were separated with a mobile phase consisting of acetonitrile/methanol/dichloromethane (70:15:15 v/v) at a flow rate of 1.8 mL/min. The retinoids were analyzed by UV absorbance at 325 nm. Values presented in Table 2 represent the sum of retinol, retinyl linoleate, retinyl oleate, retinyl palmitate, and retinyl stearate, and are expressed as micrograms of retinol equivalents per gram of tissue.

Retinol-Binding Protein (RBP) Analysis. Serum RBP was analyzed using a sensitive radioimmunoassay procedure (*30*). This procedure employs rabbit anti-rat plasma RBP antibodies and standards of purified rat plasma RBP, and has been used previously for the analysis of RBP in various mouse tissues (*31*).

Protein Determination. A modified Lowry procedure (*32*) was used for determining protein levels of cells and for measuring tissue protein levels.

Assay of Cholesteryl Ester Hydrolase and Retinyl Ester Hydrolase Activities in Tissue Homogenates, Cell Homogenates, and Cell Media. Standard CEH and REH assays were conducted according to Harrison (*6*). Briefly, for assaying REH activity, 20 μL of an appropriately diluted enzyme source was incubated at 37 °C for 60 min in an incubation mixture containing 50 mM Tris maleate, pH 8, 20 mM sodium cholate, and 0.05 μCi of retinyl[1-^{14}C]palmitate, which had been synthesized according to Azais-Braesco et al. (*33*). For CEH, the incubation mixture consisted of identical components, except that 0.05 μCi of cholesteryl-[1-^{14}C]oleate (Amersham Life Science Inc., Cleveland, OH) was used as substrate. The final concentration for both types of substrate was 10 μM.

The reaction products [[1-^{14}C]palmitic acid (for REH) and [1-^{14}C]oleic acid (for CEH)] were extracted into an alkaline, aqueous upper phase, and an aliquot of this phase was counted in a liquid scintillation counter. The amount of palmitic acid or oleic acid released was determined from the partition coefficient of those compounds and the specific activity of the substrate. For each assay, control samples without enzyme were included to correct for background activity. All assays were carried out with two concentrations of enzyme source in the range where activity was directly proportional to the amount added.

Enzyme activities were determined in an appropriate aliquot of the cell medium, or an aliquot of a cell homogenate prepared in 0.25 M sucrose using a Tenbroeck tissue grinder

Table 1: Bile Salt-Dependent and -Independent CEH and REH Activities of McA-RH7777 Cells with or without Stable Transfection of Rat Pancreatic CEL cDNA

	CE hydrolysis [pmol of fatty acid h^{-1} (mg of cell protein)$^{-1}$]		RE hydrolysis [pmol of fatty acid h^{-1} (mg of cell protein)$^{-1}$]	
enzyme source	no bs	20 mM sodium cholate	no bs	20 mM sodium cholate
cell homogenate				
McA-RH7777, contr	nd	nd	469	175
McA-RH7777, CEL tr	nd	23531	376	1073
cell-conditioned medium				
McA-RH7777, contr	nd	nd	nd	nd
McA-RH7777, CEL tr	nd	362594	nd	45964

[a] Control-transfected (contr) McA-RH7777 cells or McA-RH7777 cells stably transfected with rat pancreatic CEL cDNA (CEL tr) were grown in serum-free DMEM containing 20 μM bovine serum albumin. After 18 h, medium was collected, and cells were washed 3× with PBS, harvested, and then homogenized in 0.25 M sucrose. CEH and REH activities of media and cell homogenates were assessed in the absence of bile salt (no bs) or in the presence of 20 mM sodium cholate. Values are expressed as picomoles of [^{14}C]oleic acid (for CEH) or [^{14}C]palmitic acid (for REH) formed per hour per milligram of cell protein, and represent the average of duplicate samples. [b] bs, bile salt; nd, not detected (less than about 155 pmol h^{-1} mg^{-1} for cell homogenates and about 465 pmol h^{-1} mg^{-1} for medium).

(Wheaton Science Products, Millville, NJ). For assessing enzyme activities in mouse tissues, the pancreas and liver were homogenized in 4 mL of 0.25 M sucrose per gram of tissue using a polytron homogenizer (Brinkman Instruments, Rexdale, Ontario, Canada).

RESULTS

Bile Salt-Dependent CEH and REH Activity in Cells and Cell-Conditioned Media of Control and CEL-Transfected McA-RH7777 Cells. To confirm that active enzyme was secreted into the medium of CEL-transfected McA-RH777 cells, both CEH and REH activities were assessed in cell homogenates and in cell-conditioned media. Both bile salt-dependent CEH and REH activities were detected in the medium of CEL-transfected cells, and not in control cells (Table 1). CEH specific activity was about 8 times higher than REH activity, which is in agreement with previous studies comparing both activities using purified rat pancreatic CEL (*17*). In cell homogenates, some bile salt-dependent CEH and REH activities were detected in CEL-transfected cells, at levels of 2−6% of the value of the corresponding medium. These data are consistent with reports that CEL in liver cells is mainly destined for secretion (*3*, *19*).

Uptake of CM- or CMR-RE by CEL-Transfected and Control Transfected McA-RH7777 Cells. Figure 1 shows the time-dependent accumulation of RE from CM or CMR in control and CEL-transfected cells under conditions where enzyme had been allowed to accumulate in the culture medium for 18 h. Since hepatic bile salt-dependent CEH and REH activities are mainly secreted from the liver into the circulation, we performed these studies both in the absence of bile salt and in the presence of 10 μM sodium cholate, which corresponds with reported values for bile salt present in human plasma (*34*).

For the experiment without exogenous bile salt, cell association of retinyl palmitate from CM increased in a time-dependent manner up to 2% of the administered dose at 8 h for both control and CEL-transfected cells (Figure 1, top panel, left). For CMR (Figure 1, bottom panel, left), uptake of retinyl palmitate increased to about 7% of the dose at 8 h for both cell types. A higher uptake of RE from CMR, as compared with CM, has also been reported previously with HepG2 cells (*21*). Importantly, no statistical differences were observed in the uptake of RE from CM or CMR between

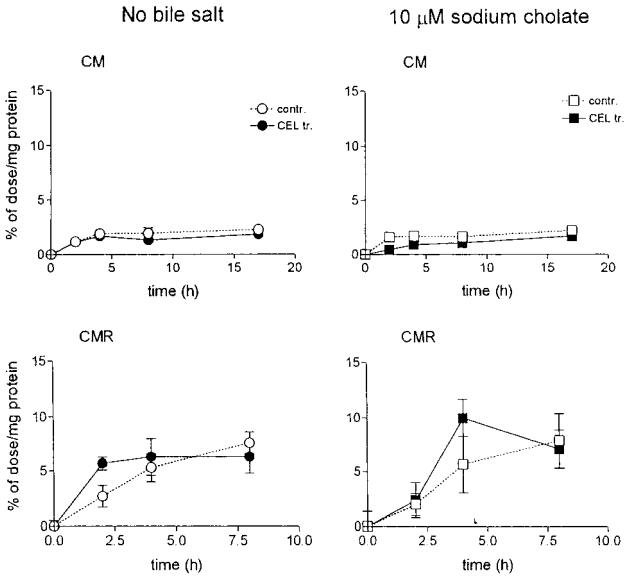

FIGURE 1: Effect of incubation time on the cell association of [^{3}H]-retinyl palmitate from rat CM or rat CMR with control transfected (contr.) and CEL-transfected (CEL tr.) McA-RH7777 cells. Top panels: Control (open symbols) or CEL-transfected (closed symbols) McA-RH7777 cells were incubated at 37 °C with CM-[^{3}H]-retinyl palmitate [2.5 μg (19 nCi) of [^{3}H]retinyl palmitate and 1490 μg of TG per well] for various periods of time, and cell-associated retinyl palmitate was determined. Incubations were carried out in the absence or presence of sodium cholate (10 μM final concentration). Values represent percentage of cell-associated retinyl palmitate per milligram of cell protein, and are the means ± SD of triplicate wells. If an error bar is not visible, it is smaller than the symbol for the point. Bottom panels: Using CMR, the cells were incubated with CMR-[^{3}H]retinyl palmitate [2.05 μg (13 nCi) of [^{3}H]retinyl palmitate and 47 μg of TG per well].

control and CEL-transfected cells. In the experiment where 10 μM sodium cholate was present during the incubation, similar results were obtained (Figure 1, top and bottom panels, right). We also did not detect any retinol formation in medium or cells of CEL-transfected or control cells at any time point studied (data not shown).

Serum Clearance and Tissue Uptake of [^{3}H]-RE from CM in WT, Heterozygous and CELKO Mice. We studied the effect of CEL deficiency on serum clearance and tissue uptake of CM-derived RE and CE. Serum clearance of both radiolabels is shown in Figure 2. For ^{3}H label and ^{14}C label, the half-times in the circulation averaged 9.8 and 12.2 min,

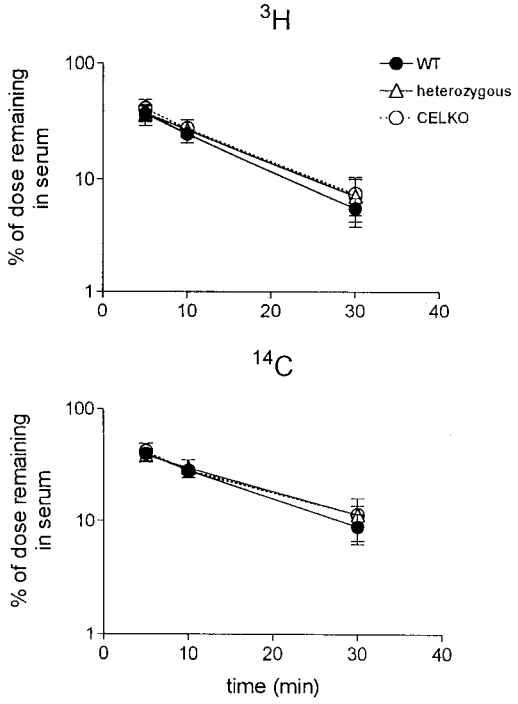

FIGURE 2: Clearance of ^{3}H and ^{14}C label from the serum of 12 h fasted WT, heterozygous, and CELKO mice after intravenous injection of rat CM containing a trace amount of [^{3}H]-RE (0.5 μCi, 30 nmol) and [^{14}C]-CE (0.12 μCi, 2.4 nmol). 5.4 mg of TG (as part of the CM) was injected in each animal. Values are expressed as percentage of total ^{3}H dose or ^{14}C dose administered remaining in the total serum compartment at each time point and are means ± SD. A total of 5 WT, 12 heterozygous, and 3 CELKO mice were examined in each group.

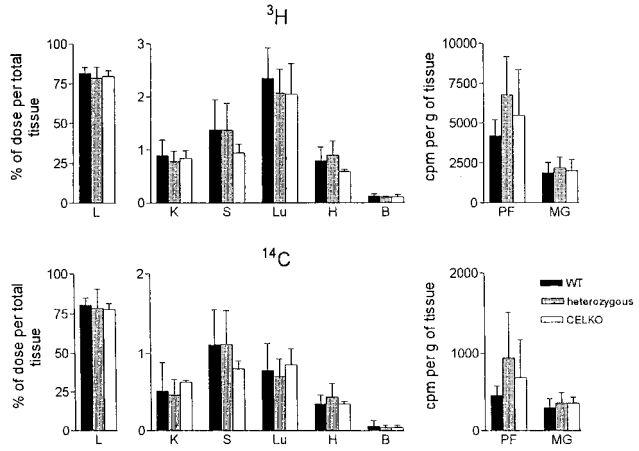

FIGURE 3: Tissue distribution of ^{3}H and ^{14}C, 30 min after intravenous injection of rat CM containing a trace amount of [^{3}H]-RE (0.5 μCi, 30 nmol) and [^{14}C]-CE (0.12 μCi, 2.4 nmol) in 12 h fasted WT, heterozygous, and CELKO mice. 5.4 mg of TG was injected in each animal. Values are expressed as percentage of total ^{3}H dose or ^{14}C dose present in each organ or tissue, after normalization for total recovery of ^{3}H and ^{14}C label, and represent means ± SD. L = liver, K = kidney, S = spleen, Lu = lung, H = heart, B = brain, PF = perirenal fat, MG = gastrocnemius muscle. A total of 5 WT, 12 heterozygous, and 3 CELKO mice were examined in each group.

respectively, for the three strains of mice, and no significant differences were observed among the three strains of mice.

Figure 3 shows the tissue distribution of ^{3}H and ^{14}C label, 30 min after injection of the CM containing [^{3}H]-RE and [^{14}C]-CE. Tissue uptake was normalized for the total ^{3}H and ^{14}C radioactivity recovered from all tissues studied, which was similar for all mice and averaged about 50−60%. As expected, liver took up the majority of ^{3}H and ^{14}C label after 30 min, averaging 80% of the recovered dose for all three mice strains. Lung, spleen, kidney, heart, and brain also accumulated ^{3}H label and ^{14}C label in lesser amounts. Because the total weights of gastrocnemius muscle or perirenal fat depot were not measured, the values for those tissues were expressed as ^{3}H cpm and ^{14}C cpm per gram of tissue. The percentage of ^{3}H and ^{14}C label present in the total serum compartment averaged 14 and 18%, respectively. No significant differences were observed in the tissue uptake of ^{3}H and ^{14}C label among WT, heterozygous, and CELKO mice in any of the tissues investigated. These data indicate that the lack of CEL expression in mice does not affect hepatic or other tissue uptake of dietary CM-RE in vivo.

Table 2: Steady-State Tissue Retinoid Levels and Serum Retinol and RBP Levels of WT, Heterozygous, and CELKO Mice

genotype parameter	WT	heterozygous	CELKO
liver			
retinoid, μg/g	970 ± 101	938 ± 102	868 ± 148
% retinol	5 ± 9	4 ± 4	2 ± 1
spleen			
retinoid, μg/g	1 ± 0	3 ± 3	2 ± 1
% retinol	44 ± 19	35 ± 17	26 ± 7
kidney			
retinoid, μg/g	4 ± 8	0.3 ± 0.1	0.4 ± 0
% retinol	52 ± 30	88 ± 3	80 ± 3
serum			
retinol, μg/dL	23 ± 5	24 ± 6	29 ± 6
RBP μg/mL	26 ± 6	24 ± 3	26 ± 5

[a] Tissue retinoid levels are expressed as micrograms of retinol equivalents per gram of tissue and represent the sum of retinol, retinyl linoleate, retinyl oleate, retinyl palmitate, and retinyl stearate. The percentage of total retinoid present as free retinol in each tissue is also presented. For each tissue, five animals of each genotype were analyzed. For serum retinol and RBP levels, the values were obtained from other litters (as >200 μL serum is required for an accurate determination of retinol level by HPLC). For those analyses, five WT mice, five heterozygous mice, and six CELKO mice were used.

The percentage of [^{3}H] retinol appearing in the liver after CM-[^{3}H]-RE uptake was also determined. No significant change in [^{3}H]retinol appearance, 30 min after injection of CM-[^{3}H]-RE, was observed in the liver of heterozygous and CELKO mice, as compared with WT mice. For these groups of mice, respectively, 13 ± 5, 10 ± 2, and 8 ± 5% of the [^{3}H]-RE taken up was present as [^{3}H]retinol.

Steady-State Tissue Retinoid Levels and Serum Retinol and RBP Levels in WT, Heterozygous, and CELKO Mice. In Table 2, the endogenous, steady-state retinoid levels of liver, spleen, kidney, and serum of WT, heterozygous, and CELKO mice (about equal numbers of male and female mice for each group) are presented. Liver, spleen, and kidney total retinoid levels were not significantly different among the three genotypes. The large mean and variance in kidney retinoid levels was due to one animal with unusually high kidney retinyl ester levels. The steady-state levels of free retinol in those tissues (expressed as percentage of total retinoid present as free retinol) did not appear to be different. Also, serum retinol and RBP levels of WT, heterozygous, and CELKO mice were all in the normal range. Hence, CEL deficiency did not appear to affect whole body retinoid homeostasis.

Bile Salt-Dependent CEH and REH Activity in Pancreas and Liver of WT, Heterozygous, and CELKO Mice. The

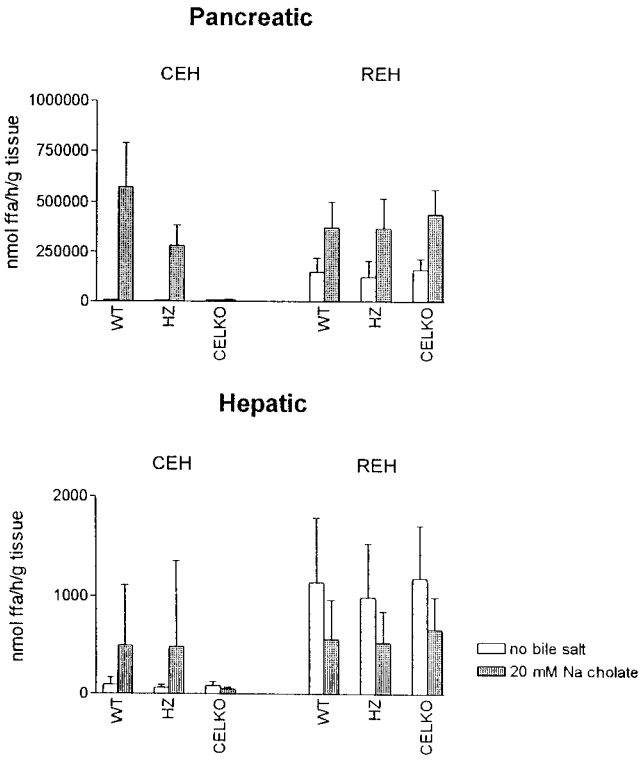

FIGURE 4: Bile salt-dependent and -independent CEH and REH activities in homogenates of pancreas and liver of WT, heterozygous, and CELKO mice. Portions of liver and pancreas were homogenized in ice-cold 0.25 M sucrose, and CEH and REH activities were determined in a small aliquot of an appropriate dilution of the tissue homogenate (typically a 1/50 dilution for liver and a 1/10 000 dilution for pancreas) in the absence and presence of 20 mM sodium cholate. Values are expressed as nanomoles of fatty acid released per hour per gram of tissue and represent the means ± SD of the number of animals studied. A total of 9 WT, 16 heterozygous, and 4 CELKO mice were examined.

above results demonstrated that uptake of RE, as well as steady-state tissue retinoid levels, was not affected by CEL deficiency. To confirm the ablation of CEL enzyme activity in the CELKO mice, both bile salt-dependent CEH and REH activities were determined in homogenates of pancreas and liver of WT, heterozygous, and CELKO mice (Figure 4). These mice represent several litters from the same heterozygous breeding pair that had been used for the CM injection experiments.

As expected, bile salt-dependent CEH activity of the pancreas of heterozygous mice was half of that of WT animals. In CELKO mice, only a trace amount was detected. In the absence of bile salt, no CEH activity was detected. A similar pattern of bile salt-dependent CEH activity was observed in liver homogenates, although the interindividual variability was much larger than in pancreas. Also, a low level of hepatic bile salt-independent CEH activity was observed in all groups (Figure 4).

To our surprise, however, in both pancreas and liver, in all three types of mice the level of REH activity in the presence of bile salt was similar. In WT mice, the observed REH activity in the pancreas is similar to CEH activity and, hence, much larger than we would expect based on the known relative CEH and REH activities of the purified rat pancreatic enzyme (*17*). This indicates that in the mouse pancreas and liver a bile salt-dependent REH is present that is distinct from CEL.

DISCUSSION

The aim of these studies was to investigate the role of CEL in the hepatic uptake and hydrolysis of dietary RE. In McA-RH7777 rat hepatoma cells, we did not observe an effect of CEL overexpression on the uptake and/or hydrolysis of CM or CMR-RE. Ji et al. (*35*), using a similar cell system for studying the role of hepatic lipase (HL) in CMR uptake, did find an enhancing effect of HL overexpression in McA-RH7777 cells on CM-and CMR-RE uptake. Those authors (*35*) speculated that HL accomplished this by a "bridging" effect; i.e., lipoprotein interaction with the cell surface was enhanced. Although CEL, like HL, has a heparin binding site, it does not have an effect equivalent to that of HL. We also studied the effect of exogenously added bile salt on CM- or CMR-RE uptake and hydrolysis in CEL-transfected and control cells. We chose a bile salt concentration known to occur in plasma, i.e., 10 μM (*34*). No enhancing effect of 10 μM sodium cholate on RE uptake was detected, nor did we detect any retinol in the medium at any time point studied. This would suggest that under physiological conditions, extracellular CEL has no impact on RE hydrolysis or on hepatic uptake of CM-derived RE.

Some evidence exists for the role of other known lipases [such as HL (*36*, *37*), lipoprotein lipase (*38*), and hormone-sensitive lipase (*39*)] in chylomicron clearance and tissue uptake of CM-derived RE in intact animals. We next asked whether CEL, which is another known RE hydrolase, expressed in and secreted by the liver (*16*, *17*), might also be involved in CM-RE metabolism in mice in vivo. Serum clearance and uptake of CM-derived RE and CE by the liver and other mouse tissues were not affected by CEL deficiency. This suggests that hepatic and/or circulatory CEL is involved neither in the rapid clearance of CM by the liver nor in the uptake of CM-derived substrate, such as RE and CE, by other tissues.

CEL deficiency had no effect on the amount of retinol formed shortly after RE uptake from CM. Also, steady-state tissue total retinoid and free retinol levels in liver and some other tissues were not different among WT, heterozygous, and CELKO mice. Retinol released from intrahepatic stores can be bound to RBP for secretion into the circulation (*40*). However, serum retinol and RBP levels, which are normally tightly controlled, were also not changed by CEL deficiency. This indicates that the absence of this lipolytic enzyme in mice does not significantly affect intrahepatic retinoid processing or hepatic secretion of the retinol−RBP complex.

In the accompanying study (*18*), a notable finding was that the intestinal processing and absorption of RE were unimpaired in CELKO mice. Thus, it is significant that we observed identical bile salt-dependent REH activities in pancreas and liver of WT, heterozygous, and CELKO mice, indicating that another enzyme activity is responsible for this bile salt-dependent REH activity. In contrast, the bile salt-dependent CEH activity in liver and pancreas of CELKO mice was absent. The simplest interpretation of our results is that CEL is the sole bile salt-dependent CEH in the mouse, but it is not the sole bile salt-dependent REH.

In this study, the absolute activity of murine pancreatic bile salt-dependent REH was the same as bile salt-dependent CEH, whereas the bile salt-dependent REH activity of purified rat pancreatic CEL is about 5 times lower than that

of bile salt-dependent CEH activity (*17*). Furthermore, in all three CEL genotypes (WT, heterozygous, or CELKO), the bile salt-dependent REH activity observed in pancreas was the same. The livers of mice of all three genotypes also had comparable levels of REH activity. This finding most likely explains why we did not observe an effect of CEL deficiency on CM-RE uptake and metabolism in mice. Thus, it is likely that another REH, distinct from CEL and active in the presence of bile salt, is present in the pancreas and the liver of mice. Future studies will focus on the identity of this enzyme activity in the mouse, and its relationship to other known lipases and retinyl ester hydrolases.

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REFERENCES

1. Wang, C.-S. (1988) *Biochem. Biophys. Res. Commun. 155*, 950−955.
2. Wang, C.-S. (1981) *J. Biol. Chem. 256*, 10198−10202.
3. Winkler, K. E., Harrison, E. H., Marsh, J. B., Glick, J. M., and Ross, A. C. (1992) *Biochim. Biophys. Acta 1126*, 151−158.
4. Wang, C.-S., and Hartsuck, J. A. (1993) *Biochim. Biophys. Acta 1166*, 1−19.
5. Harrison, E. H. (1993) *Biochim. Biophys. Acta 1170*, 99−108.
6. Harrison, E. H. (1988) *Biochim. Biophys. Acta 963*, 28−34.
7. Hernell, O., and Olivecrona, T. (1974) *Biochim. Biophys. Acta 369*, 234−244.
8. Harrison, E. H., Smith, J. E., and Goodman, D. S. (1979) *J. Lipid Res. 20*, 760−771.
9. Zolfaghari, R., Harrison, E. H., Ross, A. C., and Fisher, E. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6913−6916.
10. Chen, X., Harrison, E. H., and Fisher, E. A. (1997) *Proc. Soc. Exp. Biol. Med. 215*, 186−191.
11. 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.
12. Howles, P. N., Carter, C. P., and Hui, D. Y. (1996) *J. Biol. Chem. 271*, 7196−7202.
13. Shamir, R., Johnson, W. J., Zolfaghari, R., Lee, H. S., and Fisher, E. A. (1995) *Biochemistry 34*, 6351−6358.
14. Freed, L. M., York, C. M., Hamosh, M., Sturman, J. A., and Hamosh, P. (1986) *Biochim. Biophys. Acta 878*, 209−215.
15. Bläckberg, L., and Hernell, O. (1983) *FEBS Lett. 157*, 337−341.
16. Blaner, W. S., Prystowsky, J. H., Smith, J. E., and Goodman, D. S. (1984) *Biochim. Biophys. Acta 794*, 419−427.
17. Harrison, E. H., and Gad, M. Z. (1989) *J. Biol. Chem. 264*, 17142−17147.
18. Weng, W., Li, L., van Bennekum, A. M., Potter, S. H., Harrison, E. H., Blaner, W. S., Breslow, J. L., and Fisher, E. A. (1999) *Biochemistry 38*, 4143−4149.
19. Zolfaghari, R., Glick, J. M., and Fisher, E. A. (1993) *J. Biol. Chem. 268*, 13532−13538.
20. Goodman, D. S., Huang, H. S., and Shiratori, T. (1965) *J. Lipid Res. 6*, 390−396.
21. Lenich, C. M., and Ross, A. C. (1987) *J. Lipid Res. 28*, 183−194.
22. Gustafson, A., Alaupovic, P., and Furman, R. H. (1965) *Biochemistry 4*, 596−605.
23. Green, P. H. R., and Glickman, R. M. (1981) *J. Lipid Res. 22*, 1153−1173.
24. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911−917.
25. Forte, T. M., and Nordhausen, R. W. (1986) *Methods Enzymol. 128*, 443−457.
26. Harrison, E. H., Blaner, W. S., Goodman, D. S., and Ross, A. C. (1987) *J. Lipid Res. 28*, 973−981.
27. Kurlandsky, S. B., Gamble, M. V., Ramakrishnan, R., and Blaner, W. S. (1995) *J. Biol. Chem. 270*, 17850−17857.
28. Hultin, M., Müllertz, A., Zundel, M. A., Olivecrona, G., Hansen, T. T., Deckelbaum, R. J., Carpentier, Y. A., and Olivecrona, T. (1994) *J. Lipid Res. 35*, 1850−1860.
29. Mills, J. L., Tuomilehto, J., Yu, K. F., Colman, N., Blaner, W. S., Koskela, P., Bundle, M. S., Forman, M., Toivanen, L., and Rhodes, G. G. (1992) *J. Pediatr. 120*, 863−871.
30. Blaner, W. S. (1990) *Methods Enzymol. 189*, 270−281.
31. Wei, S., Episkopou, V., Piantedosi, R., Maeda, S., Shimada, K., Gottesman, M. E., and Blaner, W. S. (1995) *J. Biol. Chem. 270*, 866−870.
32. Markwell, M. A. K., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206−210.
33. Azais-Braesco, V., Forget, A., Mercier, M., and Grolier, P. (1992) *JAOCS 69*, 1272−1273.
34. Campbell, C. B., McGuffie, C., and Powell, L. W. (1975) *Clin. Chim. Acta 63*, 249−262.
35. Ji, Z.-S., Lauer, S. J., Fazio, S., Bensadoun, A., Taylor, J. M., and Mahley, R. W. (1994) *J. Biol. Chem. 269*, 13429−13436.
36. De Faria, E., Fong, L. G., Komaromy, M., and Cooper, A. D. (1996) *J. Lipid Res. 37*, 197−209.
37. Breedveld, B., Schoonderwoerd, K., Verhoeven, A. J. M., Willemsen, R., and Jansen, H. (1997) *Biochem. J. 321*, 425−430.
38. Blaner, W. S., Obunike, J. C., Kurlandsky, S. B., Al-Haideri, M., Piantedosi, R., Deckelbaum, R. J., and Goldberg, I. J. (1994) *J. Biol. Chem. 269*, 16559−16565.
39. Wei, S., Lai, K., Patel, S., Piantedosi, R., Shen, H., Colantuoni, V., Kraemer, F. B., and Blaner, W. S. (1997) *J. Biol. Chem. 272*, 14159−14165.
40. Soprano, D. R., and Blaner, W. S. (1994) in *The Retinoids: Biology, Chemistry and Medicine* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds.) pp 257−281, Raven Press, New York.

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