JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Cholesteryl Ester Species Differently Elevate Plasma Cholesterol in Hamsters

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ABSTRACT: This study was to examine the effect of free cholesterol (C) and individual cholesteryl ester (CE) species, namely cholesteryl palmitate (CP), cholesteryl stearate (CS), cholesteryl oleate (CO), and cholesteryl linoleate (CL) on plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), non-HDL-C, and triacylglycerols (TG) in hamsters. Results showed that addition of dietary CE species into diet at 0.1% differently raised plasma TC concentrations, with CO elevating plasma TC to 331 mg/dL, while CS raised plasma TC only to 220 mg/dL. It was found that CS was a poor substrate of pancreatic cholesterol esterase, while CO was a good substrate. The fecal analysis showed CS-fed hamsters had the highest fecal cholesterol concentration, while RT-PCR analysis found CS feeding was associated with down-regulations of intestinal Niemann-Pick C1 like 1 (NPC1L1) and acyl-CoA: cholesterol acyltransferase 2 (ACAT2) as well as microsomal triacylglycerol transport protein (MTP). It was therefore concluded that the plasma cholesterol-raising activity of CE species was partially governed by their hydrolysis rates in the intestine, and the relative low raising activity associated with CS was mediated by down-regulation of intestinal NPC1L1, ACAT2, and MTP.

KEYWORDS: *HMG-CoA reductase, cholesterol, HDL cholesterol, NPC1L1, ACAT 2, MTP*

INTRODUCTION

A significant correlation has been demonstrated between daily cholesterol intake and death rate from coronary heart disease in 24 countries.[1] On average, males in the U.S. consume 307 mg of cholesterol per day, and females consume 225 mg.[2] In Hong Kong, men have a cholesterol intake of 399 mg, while women have 285 mg.[3] In Singapore, males and females daily consume 384 and 295 mg cholesterol, respectively.[4] In Beijing, China, daily cholesterol intake reaches an average of 380 mg.[5] In the EU, people have an average cholesterol intake of 450 mg per day.[6,7] There is a marked heterogeneity among humans in their response of plasma cholesterol concentration to dietary cholesterol consumption.[8] In general, earlier investigations clearly demonstrated plasma cholesterol concentration in humans had a moderate elevation under wide-ranging amounts of dietary cholesterol intake.[9−12] A clear association between dietary cholesterol and plasma cholesterol concentrations has also been observed in animals, including hamsters, rabbits, guinea pig, swine, and primates.[13−15]

Cholesterol is present in the human diet in two major forms, namely free cholesterol and cholesteryl esters (CE). It is known that CE constitutes two-thirds of the total cholesterol in the plasma and approximately one-third of the total cholesterol in diets.[16,17] It is estimated that the total cholesterol intake, including free cholesterol and CE is about 200−500 mg/day. In this regard, humans daily consume about 60−150 mg CE. Most CE present in diets is of animal origin and consists of a mixture of CE species with esterification of various fatty acids, mainly including cholesteryl palmitate (CP), cholesteryl stearate (CS), cholesteryl oleate (CO), and cholesteryl linoleate (CL).

Absorption of cholesterol and CE is a complex process. First, cholesterol absorption starts with pancreatic cholesterol esterase (PCE), which is responsible for the hydrolysis of CE and mediates cholesterol absorption.[18,19] Second, a transporter protein, Niemann-Pick C1 like 1 (NPC1L1), transports the free cholesterol from the lumen into enterocytes. Third, intestinal acyl-CoA: cholesterol acyltransferase 2 (ACAT2) then converts the free cholesterol to CE. Microsomal triacylglycerol transport protein (MTP) packs CE into chylomicrons (CM), which is then transferred into the blood through the lymphatic system.[20,21] Lastly, the ATP-binding cassette transporters subfamily G member 5 and 8 (ABCG5/8) carry the unabsorbed free cholesterol left in the enterocytes back to the lumen for excretion.[22,23]

Although extensive research has investigated the effect of dietary cholesterol on cardiovascular health, very little is known on how dietary CE species affect plasma cholesterol concentrations. The present study was therefore the first to (i) investigate the effect of dietary CE species on plasma lipoprotein

Received: September 4, 2013
Revised: October 23, 2013
Accepted: October 23, 2013
Published: October 23, 2013

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Table 1. Change in Food Consumption, Body Weight and Relative Organ Weights in Hamsters Fed the Control Diet Containing 2.6 mmol Cholesterol (C) or One of the Four Experimental Diets Containing 2.6 mmol Cholesteryl Palmitate (CP), Cholesteryl Stearate (CS), Cholesteryl Oleate (CO), Cholesteryl Linoleate (CL), Respectively, per kg Diet[a]

	C	CP	CS	CO	CL	p value
food intake (g)						
	9.3 ± 0.3	9.5 ± 0.6	9.0 ± 0.6	8.8 ± 0.4	8.9 ± 0.4	0.18
body weight (g)						
initial	114.8 ± 8.7	114.0 ± 4.9	115.3 ± 5.5	114.8 ± 7.3	113.3 ± 2.3	0.96
final	126.7 ± 8.1^{b}	137.3 ± 13.2^{a}	128.1 ± 9.2^{ab}	131.9 ± 12.0^{ab}	132.1 ± 10.1^{a}	0.22
organ weight (% body weight)						
liver	5.16 ± 0.41	5.19 ± 0.17	4.95 ± 0.41	5.09 ± 0.34	5.04 ± 0.38	0.57
kidney	0.81 ± 0.05	0.80 ± 0.05	0.79 ± 0.06	0.82 ± 0.05	0.78 ± 0.04	0.58
heart	0.36 ± 0.03	0.35 ± 0.03	0.36 ± 0.02	0.36 ± 0.02	0.35 ± 0.02	0.42
epididymal fat	1.66 ± 0.29	1.76 ± 0.26	1.65 ± 0.25	1.75 ± 0.15	1.79 ± 0.26	0.67
perirenal fat	1.09 ± 0.23^{ab}	1.17 ± 0.33^{ab}	1.04 ± 0.25^{b}	1.16 ± 0.20^{ab}	1.31 ± 0.37^{a}	0.30

[a]Data were expressed as mean ± SD; n = 10 each group; Means at the same row with different superscript (a, b) differ significantly at $p < 0.05$.

profiles; and (ii) study the interaction of CE species with gene expression of NPC1L1, ACAT2, MTP, and ABCG5/8 using hamsters as a model.

■ MATERIALS AND METHODS

Materials. Cholesterol was purchased from Sigma Chemical (St. Lois, MO, U.S.), whereas CP, CS, CO, and CL were obtained from Atomax Chemical Co., Ltd. (Shenzhen, China). The purity of each chemical was checked on a fused silica capillary column (SAC-5, 30 m × 0.25 mm, i.d.; Supelco, Bellefonte, PA, U.S.) using a Shimadzu GC-14 B Gas−Liquid Chromatograph equipped with a flame ionization detector. It was found that the purity of cholesterol, CP, CS, CO, and CL was 95%, 97%, 97%, 91%, and 95%, respectively. PCE was purchased from Oriental Yeast Co., Ltd. (Osaka, Japan).

Diets. The five diets were prepared. All diet ingredients were purchased from Harlan Teklad (Madison, WI, U.S.) except for lard, which was bought from the local market. DL-Methionine was purchased from Sigma Chemical (St. Lois, MO, U.S.). The basal diet per kg contained 508 g corn starch, 242 g casein, 50 g lard, 119 g sucrose, 40 g mineral mix, 20 g vitamin mix, 1 g DL-methionine. After justification of the purity of cholesterol and CE, the control diet was prepared by adding 2.6 mmol cholesterol (C) per kg diet (equivalent to 0.1% cholesterol), while the other four experimental diets were prepared by adding 2.6 mmol CP, 2.6 mmol CS, 2.6 mmol CO, and 2.6 mmol CL into the basal diet, respectively. One liter of gelatin solution (20 g/L) was mixed with 200 g diet. The gelatinized diets were cut into approximate 10 g cubes and stored frozen at −20 °C.

Hamsters. Golden hamsters (n = 50; body weights = 110−120 g) were randomly divided into five groups (n = 10). Experimental procedures were approved by the Animal Experimental Ethical Committee, The Chinese University of Hong Kong. All hamsters were housed in wire-bottomed cages at 23 °C in an animal room with 12-h light-dark cycle. Diets and water were given ad libitum. All hamsters were weighed and their total feces per cage were saved weekly. After fasting overnight, 0.5 mL blood sample was obtained from the retro-orbital sinus into a heparinized capillary tube under inhalational anesthesia of isoflurane (100%) at the beginning of week 1 and the end of week 3 and 6. Following the last blood sample collection at week 6, all of the hamsters were killed by carbon dioxide suffocation. The liver, heart, kidney, epididymal, and perirenal adipose tissues and aorta were removed, washed in saline, and weighed. The first 10 cm of duodenum was discarded, and the next 30 cm of the small intestine was kept. All tissue samples were flash frozen in liquid nitrogen and stored at −80 °C until analysis.

Plasma Lipoprotein Quantification. Two commercially available enzymatic kits from Infinity (Waltham, MA, U.S.) and Stanbio Laboratories (Boerne, TX, U.S.) were used to quantify plasma total cholesterol (TC) and total triacylglycerols (TG), respectively. To quantify plasma HDL cholesterol (HDL-C), a commercial solution (Stanbio Laboratories) containing phosphotungstic acid and magnesium chloride were added into plasma to precipitate the very low-density lipoprotein (VLDL) and LDL cholesterol (LDL-C). HDL-C in the supernatant was measured similarly as it was done for TC. Non-HDL cholesterol (non-HDL-C) was calculated by deducting HDL-C from TC.

Liver Cholesterol Analysis. Cholesterol in the liver was quantified using a method as we previously described.[24,25] In brief, about 300 mg liver sample was weighed and homogenized with 5α-cholestane being added as an internal standard. The liver total lipids were extracted into a solvent mixture of methanol/chloroform (2:1, vol/vol). After the solvents were evaporated under nitrogen gas, the liver total lipids were mildly saponified. The unsaponified sterols containing cholesterol in sample was converted to its trimethylsilyl-ether (TMS) derivative before the GC analysis.

Fecal Sterol Analysis. Fecal total and individual neutral and acidic sterols were analyzed as we previously described.[25,26] Fecal samples were first freeze-dried and ground into powder. In each analysis, 500 mg fecal powder was saponified with addition of 5α cholestane and hyodeoxycholic acid as internal standards for quantification of fecal neutral and acidic sterols, respectively. Total neutral sterols were extracted into cyclohexane and converted into their TMS derivatives. The acidic sterols in the remaining aqueous layer were also saponified, extracted and converted into their corresponding TMS derivatives. The individual neutral and acidic sterol TMS derivatives were analyzed in a fused silica capillary column (SAC-5, 30 m × 0.25 mm, i.d.; Supelco, Bellefonte, PA, U.S.) using a Shimadzu GC-14 B Gas−Liquid Chromatograph equipped with a flame ionization detector.

Real-Time PCR Analysis. Real-time PCR was employed to quantify mRNA of intestinal NPC1L1, ABCG5/8, ACAT2, and MTP.[27,28] In brief, Tizol Reagent (Invitrogen, Carslbad, CA, U.S.) was used to extract total RNA from the intestine followed by converting total RNA to its complementary DNA (cDNA) using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, U.S.). Real-time PCR analysis was carried out on a Fast Real-time PCR System 7500 (Applied Biosystems). mRNA of intestinal NPC1L1, ABCG5, ABCG8, ACAT2, MTP, and 18S was measured using SYBR green as fluorophore.[29] Data were analyzed using the Sequence Detection Software version 1.3.1.21 (Applied Biosystems).

Measurement of Intestinal ACAT Activity. Intestinal ACAT activity was measured as we previously described.[24,30] In brief, the intestinal microsome was prepared followed by adding cholesterol into 45% (w/v) 2-hydroxypropyl β-cyclodextrin aqueous solution and then incubated in a 37 °C water bath for 5 min. The reaction was initiated by adding an assay reagent of 0.52 nmol of [^{14}C] oleoyl-Coenzyme A (0.03 μCi), 7.48 nmol of nonradioactive oleoyl-Coenzyme A and 10 nmol of fatty acid-free bovine serum albumin. After 20 min, the reaction was stopped by adding 4.8 mL of chloroform/methanol mixture (2:1, v/v) and 1 mL saline. After addition of 10 μg [^{3}H] cholesterol oleate (0.002 μCi), the mixture was centrifuged at 800g for 10 min at 4 °C, and the

Table 2. Change in Hepatic Cholesterol and Plasma Total Cholesterol (TC), Total Triacylglycerols (TG), High Density Lipoprotein Cholesterol (HDL-C), and non-HDL Cholesterol (non-HDL-C) in Hamsters Which Fed the Control Diet Containing 2.6 mmol Cholesterol (C) or One of the Four Experimental Diets Containing 2.6 mmol Cholesteryl Palmitate (CP), Cholesteryl Stearate (CS), Cholesteryl Oleate (CO), Cholesteryl Linoleate (CL), Respectively, per kg Diet[a]

	C	CP	CS	CO	CL	*p* value
week 0						
TC (mg/dL)	130.2 ± 14.6	132.4 ± 8.6	131.6 ± 15.2	130.5 ± 14.0	131.8 ± 11.0	0.99
HDL-C (mg/dL)	88.7 ± 7.4	91.3 ± 4.6	91.4 ± 5.1	91.2 ± 5.34	90.1 ± 6.6	0.81
non-HDL-C (mg/dL)	41.5 ± 9.6	41.1 ± 7.8	40.3 ± 13.2	39.3 ± 10.5	41.7 ± 11.8	0.99
non-HDL-C/HDL-C	0.47 ± 0.10	0.45 ± 0.09	0.44 ± 0.14	0.43 ± 0.10	0.47 ± 0.15	0.94
HDL-C/TC	0.68 ± 0.05	0.69 ± 0.04	0.70 ± 0.06	0.70 ± 0.05	0.69 ± 0.07	0.90
TG (mg/dL)	88.9 ± 12.5	99.0 ± 18.5	93.3 ± 19.6	100.0 ± 25.7	95.3 ± 15.5	0.69
week 3						
TC (mg/dL)	286.7 ± 33.4[ab]	288.7 ± 52.8[ab]	253.5 ± 46.3[b]	296.2 ± 61.0[a]	285.3 ± 25.0[ab]	0.28
HDL-C (mg/dL)	136.8 ± 11.5	122.8 ± 14.5	120.5 ± 23.6	130.9 ± 8.2	138.4 ± 23.9	0.10
non-HDL-C (mg/dL)	149.9 ± 23.0	165.9 ± 38.9	133.0 ± 20.4	154.3 ± 20.2	157.8 ± 60.1	0.50
non-HDL-C/HDL-C	1.09 ± 0.10	1.34 ± 0.18	1.16 ± 0.49	1.18 ± 0.46	1.14 ± 0.14	0.55
HDL-C (mg/dL)	0.48 ± 0.02	0.43 ± 0.04	0.49 ± 0.13	0.44 ± 0.13	0.48 ± 0.03	0.55
TG (mg/dL)	177.6 ± 58.7[bc]	216.6 ± 76.0[ab]	152.6 ± 57.7[c]	159.6 ± 48.5[bc]	210.2 ± 71.1[ab]	0.10
week 6						
TC (mg/dL)	290.7 ± 33.1[b]	274.1 ± 33.2[b]	220.6 ± 26.6[c]	331.3 ± 34.4[a]	302.3 ± 36.1[ab]	<0.01
HDL-C/TC	150.8 ± 14.4[ab]	141.7 ± 17.1[b]	122.5 ± 15.5[c]	164.1 ± 26.6[a]	146.7 ± 12.1[b]	<0.01
non-HDL-C (mg/dL)	139.9 ± 20.1[bc]	132.4 ± 18.8[c]	98.1 ± 16.5[d]	167.2 ± 24.2[a]	155.6 ± 25.5[ab]	<0.01
non-HDL-C/HDL-C	0.93 ± 0.07[ab]	0.94 ± 0.10[ab]	0.81 ± 0.16[b]	1.06 ± 0.36[a]	1.06 ± 0.12[a]	0.03
HDL-C/TC	0.52 ± 0.02[b]	0.52 ± 0.03[b]	0.56 ± 0.04[a]	0.49 ± 0.06[b]	0.49 ± 0.03[b]	<0.01
TG (mg/dL)	221.5 ± 75.1[ab]	234.5 ± 94.3[ab]	165.5 ± 67.3[b]	279.2 ± 88.6[a]	297.3 ± 95.4[a]	0.02
liver						
cholesterol (mg/g)	46.0 ± 6.3[a]	25.1 ± 7.8[cd]	20.6 ± 4.5[d]	41.6 ± 6.6[a]	38.8 ± 4.1[b]	<0.01

[a]Data were expressed as mean ± SD; n = 10 each group; Means at the same row with different superscript (a, b, c) differ significantly at $P < 0.05$.

lower organic layer was collected and transferred to a new tube and evaporated under a gentle nitrogen stream until dryness. Ten μg of cholesteryl oleate in 50 μL of chloroform was then added, and the tube was vortexed thoroughly. The resuspension was then spotted on a thin-layer silica gel plate (Merck, NJ, U.S.) and developed in hexane/ethyl acetate/acetic acid (80:20: 1, v/v) for 45 min. The band corresponding to cholesterol oleate was cut off and transferred into a scintillation vial. Ten mL of OptiPhaseHiSafe 2 scintillation fluid (Perkin-Elmer) was added into the vial and incubated with agitation overnight. Radioactivity was then measured in a LS 6500 scintillation counter (Beckman), and the data were calculated based on [^{3}H] recovery.

Enzymatic Assay of Pancreatic Cholesterol Esterase (PCE). Each cholesterol ester (0.2 g) was purified using silica gel column with a solvent of hexane/diethyl ether (50:1, vol/vol) as a mobile phase. To conduct the assay, the reaction mixture was prepared by mixing 2.5 mL of (A) 0.1 M of sodium phosphate buffer with pH 7.0 containing 0.91 mM 4-amino-antipyrine (Wako, Japan) and 6.0 mM phenol (Wako, Japan), (b) 0.2 mL of 0.66 M sodium cholate (Sigma, St Louis, MO, U.S.), (C) 0.1 mL of 1.0 mg/mL horseradish peroxidase (Wako, Japan), and (D) 0.05 mL of 35 IU/mL cholesterol oxidase (Wako, Japan). The reaction mixture was then mixed with 0.5 mL of 10% NIKKOL BL-9EX containing 4.0 mM each cholesteryl ester. The hydrolysis was initiated by adding 0.05 mL of 0.1 IU/mL pancreatic cholesterol esterase (Wako, Japan), reaching a final volume 3.4 mL at 37 °C. The rate of hydrolysis of each cholesteryl ester was measured spectrophotometrically at a wavelength of 505 nm.

Statistics. Data were expressed as mean ± standard deviation (SD), n = 10. The significance of difference was assessed using ANOVA in SigmaStat Advisory Statistical Software (SigmaStat version 14.0, SPSS Inc., Chicago, U.S.). Differences among five groups were considered significant when $P < 0.05$.

RESULTS

Food Intake, Body and Organ Weight. No differences in food intake and initial body weights were observed among the five groups. The final body weights did not differ except that CP group weighed heavier than the C group (Table 1). When the organ weights were expressed as the percentage of total body weight, no significant differences in the weights of liver, heart, kidney, and epididymal adipose tissue were seen among the five groups. However, the perirenal adipose pad in CL-fed group was greater than that of CS-fed hamster (Table 1).

Plasma TC, HDL-C, TG, and non-HDL-C/HDL-C. Hamsters were fed the rodent chow diet before they were switched to their respective experimental diets. Across the 6 week period on the experimental diet, C, CO, and CL groups showed a trend of increase, while CP and CS groups demonstrated a trend of increase from week 0 to week 3, followed by a decrease in plasma TC (Table 2). At the end of week 6, it was found that dietary CE species differently raised plasma TC and non-HDL-C concentrations with CS being the least and CO being the most. The difference was magnificent, with TC in the CO group reaching 331.3 mg/dL, while that in the CS group was only 220.6 mg/dL. Similarly, plasma TG varied significantly with CL group, having the highest TG concentration of 297.3 mg/dL, whereas the CS group had the least TG concentration of 165.5 mg/dL (Table 2).

Liver Cholesterol. Hepatic cholesterol concentrations differed significantly among the five groups. C group had the highest hepatic cholesterol concentration of 46.0 mg/g, whereas CS hamsters had the lowest hepatic cholesterol of 20.6 mg/dL (Table 2).

Fecal Total Sterol Excretion and Apparent Sterol Balance. To simplify the presentation, only data on fecal excretion of sterols at week 6 are presented. Fecal excretion of cholesterol by CS group was the greatest, followed by CP, CL, CO, and C in a decreasing order (Table 3). Total fecal neutral

Table 3. Cholesterol Intake (mg/day), Fecal Individual Neutral and Acidic Sterols and Their Derivatives in Hamsters Fed the Control Diet Containing 2.6 mmol Cholesterol (C) or One of the Four Experimental Diets Containing 2.6 mmol of Cholesteryl Palmitate (CP), Cholesteryl Stearate (CS), Cholesteryl Oleate (CO), Cholesteryl Linoleate (CL), Respectively, per kg Diet[a,b,c]

	C	CP	CS	CO	CL	*p* value
cholesterol intake (CI)						
	8.61 ± 0.45	9.41 ± 0.57	8.57 ± 0.80	8.65 ± 0.65	8.95 ± 0.32	0.16
fecal neutral sterols (NS)						
coprostanol	0.67 ± 0.12	0.66 ± 0.40	0.49 ± 0.10	0.68 ± 0.22	0.71 ± 0.18	0.58
coprostanone	0.09 ± 0.05	0.06 ± 0.02	0.09 ± 0.04	0.07 ± 0.01	0.06 ± 0.01	0.37
cholesterol	0.43 ± 0.22[c]	1.54 ± 0.33[b]	3.73 ± 0.63[a]	0.79 ± 0.16[c]	0.85 ± 0.19[c]	<0.01
dihydrocholesterol	0.20 ± 0.07	0.21 ± 0.03	0.20 ± 0.06	0.21 ± 0.04	0.23 ± 0.03	0.88
campersterol	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.21
β-sitosterol	0.03 ± 0.03	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.80
total	1.45 ± 0.24[c]	2.53 ± 0.29[b]	4.56 ± 0.78[a]	1.81 ± 0.42[c]	1.90 ± 0.10[c]	<0.01
fecal acidic sterols (AS)						
lithocholic acid	1.37 ± 0.62	2.13 ± 1.04	1.60 ± 0.68	1.02 ± 0.40	1.49 ± 0.72	0.22
deoxycholic acid	0.29 ± 0.15	0.24 ± 0.10	0.30 ± 0.21	0.27 ± 0.17	0.24 ± 0.12	0.95
chenodeoxycholic + cholic acid	0.27 ± 0.11	0.38 ± 0.20	0.43 ± 0.27	0.30 ± 0.06	0.40 ± 0.20	0.63
ursodeoxycholic acid	0.08 ± 0.02	0.14 ± 0.07	0.09 ± 0.03	0.08 ± 0.03	0.09 ± 0.04	0.13
total	2.01 ± 0.60	2.90 ± 1.19	2.42 ± 0.60	1.67 ± 0.44	2.21 ± 0.66	0.15
apparent sterol absorption % (CR/CI)	59.8 ± 6.9[a]	42.4 ± 12.5[b]	18.5 ± 9.9[c]	59.6 ± 7.9[a]	53.8 ± 10.0[ab]	<0.01

[a]Cholesterol retention (CR) = (cholesterol intake)-(fecal cholesterol + its derivative + fecal bile acids). [b]Apparent sterol absorption = [(cholesterol intake)-(fecal cholesterol + its derivative + fecal bile acids)]/cholesterol intake. [c]Data were expressed as mean ± SD; $n = 10$ each group; Means at the same row with different superscript (a, b, c) differ significantly at $P < 0.05$.

sterols had a similar excretion pattern with CS group excreting the most (4.56 mg/day), whereas C group excreted the least (1.45 mg/day). Regarding the excretion of acidic sterols, CP group excreted the most (2.90 mg/day), whereas the CO group excreted the least (1.67 mg/day). The difference in excretion of total acidic sterols among the five groups was much smaller than that in the excretion of total neutral sterols. The apparent sterol absorption could be calculated using an equation: Apparent sterol absorption = [(cholesterol intake) − (fecal neutral sterols + acidic sterols)]/cholesterol intake. At week 6, the difference in cholesterol absorption among the five groups was magnificent, with CS being only 18.5% and C being 59.8% (Table 3).

mRNA of intestinal NPC1L1, ABCG5/8, ACAT2, and MTP. Real-Time PCR analysis demonstrated that CS group had the least mRNA of intestinal NPC1L1, whereas C and CL hamsters had the highest mRNA levels (Figure 1). Similarly, the mRNA of intestinal MTP in CS was also the least, while that in C and CL was the highest. CS group had the least mRNA of intestinal ACAT2. In contrast, C group had the highest mRNA of intestinal ACAT2.

Intestinal ACAT Enzymatic Activity. Feeding C, CP, CS, CO, and CL had varying effect on the intestinal ACAT activity. However, only significant differences were seen between the CS and CL groups, with the former having the least ACAT activity, while the latter had the highest ACAT enzymatic activity (Figure 2).

Hydrolysis of Individual CE Species by PCE. PCE is responsible for hydrolyzing CE before it can be absorbed. PCE demonstrated a huge difference in hydrolyzing different CE species to free cholesterol in vitro with being most effective for the hydrolysis of CO and least effective for the hydrolysis of CS (Figure 3).

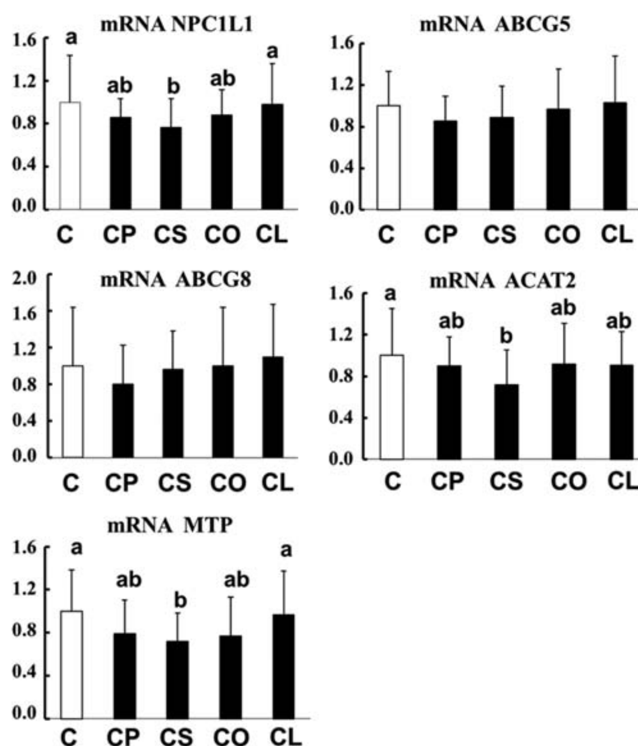

Figure 1. Effect of cholesterol (C), cholesteryl palmitate (CP), cholesteryl stearate (CS), cholesteryl oleate (CO), and cholesteryl linoleate (CL) on mRNA levels of intestinal Niemann-Pick C1 like 1 (NPC1L1), acyl coenzyme A: cholesterol acyltransferase 2 (ACAT2), microsomal triacylglycerol transport protein (MTP), ATP binding cassette transporters (ABCG5 and ABCG8) in hamsters. Data are normalized with cyclophilin. Values are expressed as means ± SD ($n = 10$) with those for Cholesterol group being arbitrarily taken as one. Bars with different letters (a, b) are significantly different at $p < 0.05$.

DISCUSSION

The present study investigated the effect of individual CE species on plasma TC, non-HDL-C, HDL-C, and TG concentrations in hamsters compared with that of free cholesterol. We had the following observations. First, dietary CE species differently raised plasma TC concentrations, with CO being the most effective and

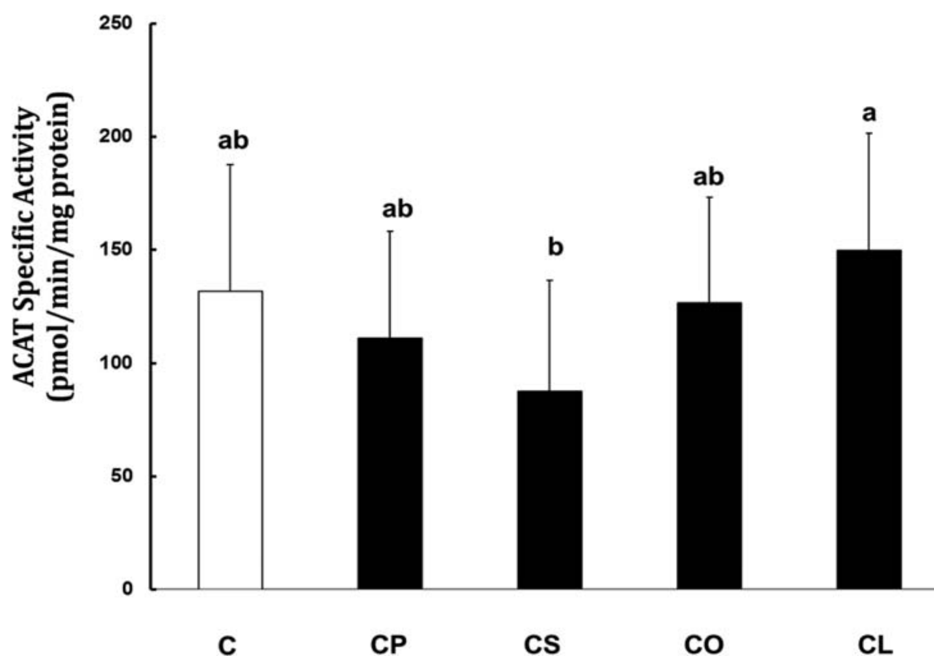

Figure 2. Effect of cholesterol (C), cholesteryl palmitate (CP), cholesteryl stearate (CS), cholesteryl oleate (CO), and cholesteryl linoleate (CL) on intestinal acyl coenzyme A: cholesterol acyltransferase (ACAT) activity in hamsters. Values are expressed as means ± SD ($n = 10$). Bars with different letters (a, b) are significantly different at $p < 0.05$.

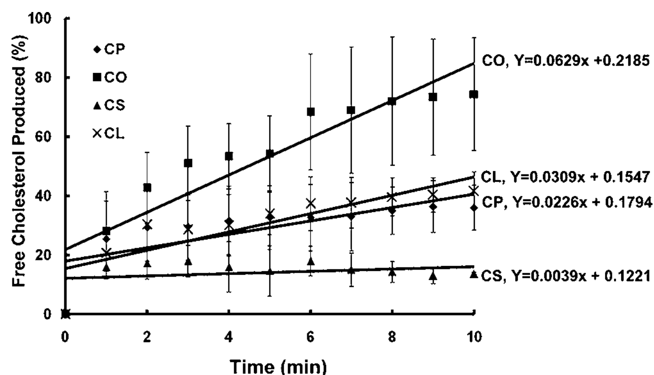

Figure 3. Pancreatic cholesterol esterase (PCE)-dependent hydrolysis rate of cholesteryl palmitate (CP), cholesteryl stearate (CS), cholesteryl oleate (CO), and cholesteryl linoleate (CL) in vitro. The details on assay solution were described in Materials and Methods.

CS being the least effective (Table 2). Second, it was unexpected that CE species with unsaturated fatty acids, oleic and linoleic acid, raised plasma TC higher than CE species with saturated fatty acids, palmitic and stearic acids, indicating that the unsaturation of the fatty acid moiety had no suppressive effect on the plasma TC-raising activity of a CE species. Third, the difference in raising plasma TC by four CE species was magnificent, with the plasma TC in the CO group being elevated to 331 mg/dL, while that in the CS group was elevated only to 220 mg/dL. We are unaware of any reports that had examined either the cholesterol-raising activity of individual CE species in diet or the effect of CE species on the transporters and enzymes involved in cholesterol absorption, so that we have nothing with which to directly compare our present data. In view of 200−500 mg total cholesterol being present in human diets,[2−8] with up to 30% being in the form of CE,[16,17] the impact of dietary CE species on plasma TC in humans could be significant. It was therefore deemed necessary to study the underlying mechanism by which CE species exhibited such varying plasma cholesterol-raising activity.

We hypothesize that dietary individual CE species have a varying cholesterol absorption rate, thus leading to a significant difference in plasma TC-raising activity. The hypothesis is supported by the following evidence. First, CE cannot be absorbed directly and requires prior hydrolysis before absorption. In this connection, the CE-hydrolyzing enzyme, namely PCE, demonstrated a huge difference in hydrolyzing CE species to free cholesterol in vitro, being most effective for CO and least effective for CS, suggesting that CS was a poor substrate and CO was a good substrate for PCE (Figure 3), even though the mechanism for the difference in the hydrolysis rate is unknown. The present results are in agreement with that of Brown et al.[31] who studied the substrate specificity and found that PCE preferred CE in an order of CO, CP, and CS. Second, the fecal cholesterol analysis showing that plasma TC concentration was negatively associated with the fecal cholesterol concentrations (Tables 2 and 3). To be specific, the CS-fed group had the highest concentrations of fecal cholesterol and total neutral sterols, indicating that CS had the lowest cholesterol absorption rate. This was further evidence that the CS group had an apparent sterol absorption rate much smaller than those of the other four groups. Thus, among the four CE species, dietary CS was expected to have the least plasma TC-raising activity and vice versa for CO.

Results from Real-time PCR analysis of mRNA further supported the hypothesis that the difference of CE species in plasma-TC raising activity was attributable to their varying hydrolysis rates by the intestinal PCE. CE cannot be directly absorbed and must be first hydrolyzed to free cholesterol before absorption. The cholesterol absorption is governed by two types of intestinal transporters, namely NPC1L1 and ABCG 5/8. The former is an influx transporter responsible for channeling free cholesterol from the intestinal lumen into enterocytes, whereas the latter are the efflux transporters responsible for shuttling

some unesterified cholesterol back to the lumen for excretion. In sequence, the enzyme ACAT2 and the transport protein MTP are also essential in regulation of cholesterol absorption, with the former promoting intracellular sterol esterification in the enterocytes, while MTP is responsible for the chylomicrons assembly. Results clearly showed that the CS group had a down-regulation of mRNA NPC1L1, ACAT2, and MTP among the five groups. This was consistent with the observation that the CS group had the least intestinal ACAT2 enzymatic activity.

In conclusion, humans daily consume about 200−500 mg cholesterol, with approximately 30% being present in the form of CE (Figure 4). Although the data could not be directly extrapolated to what happens in humans, the present study is the first to demonstrate that for a given amount, CE species had varying plasma cholesterol-raising activity. It was concluded that CS species had the least plasma TC-raising activity, most likely mediated by its poor hydrolysis rate by PCE and down-regulation of NPC1L1, ACAT2, and MTP gene expression.

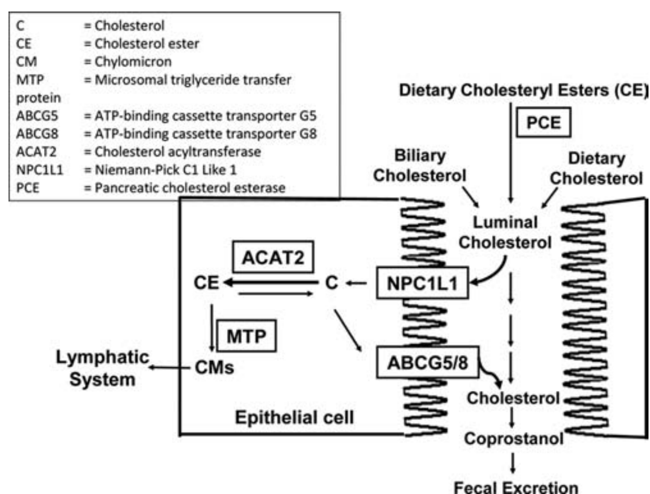

Figure 4. Suggested absorption route of dietary cholesterol (C) and cholesteryl esters (CE). CE in diet is hydrolyzed by pancreatic cholesterol esterase (PCE) to release free cholesterol in the intestine. Cholesterol, either from diet or bile, is transported into enterocytes via intestinal Niemann-Pick C1 like 1 (NPC1L1). Intestinal acyl coenzyme A:cholesterol acyltransferase 2 (ACAT2) esterifies cholesterol to form cholesteryl ester (CE), which is packed with microsomal triacylglycerol protein (MTP) into chylomicrons (CM) and transferred into blood through the lymphatic system. ATP binding cassette transporters (ABCG5 and ABCG8) return the unabsorbed cholesterol to the lumen of the intestine for excretion.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This project was supported by Hong Kong GRF grant (Project Number CUHK 461112).

ABBREVIATIONS USED

ABCG5/8, ATP-binding cassette transporters subfamily G member 5 and 8; ACAT 2, acyl-CoA: cholesterol acyltransferase 2; C, cholesterol; CL, cholesteryl linoleate; CO, cholesteryl oleate; CP, cholesteryl palmitate; CS, cholesteryl stearate; CM, chylomicrons; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MTP, Microsomal triacylglycerol transport protein; non-HDL-C, nonhigh-density lipoprotein cholesterol; NPC1L1, Niemann-Pick C1 like 1; PCE, pancreatic cholesterol esterase; TC, total cholesterol

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