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Frequent Cholesterol Intake Up-regulates Intestinal NPC1L1, ACAT2, and MTP

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Dietary cholesterol elevates plasma total cholesterol (TC) level. However, no study to date has examined how cholesterol intake frequency interacts with the gene of sterol transporters, receptors, and enzymes involved in cholesterol metabolism. Thirty-three hamsters were divided into three groups with the control hamsters being given daily 9 mg of cholesterol in the diet (CD), whereas the second group being gavage-administered 3 mg of cholesterol three times per day (C-3) and the third group being gavage-administered 9 mg of cholesterol one time per day (C-1). The experiment lasted for 6 weeks. The hamsters were killed under carbon dioxide suffocation. Data demonstrated that plasma TC, non-high-density lipoprotein cholesterol, and triacylglycerols were elevated with the increasing cholesterol intake frequency. Western blotting analyses revealed that the intake frequency had no effect on protein mass of hepatic sterol regulatory element binding protein-2, liver X receptor-α, 3-hydroxy-3-methylglutaryl-CoA reductase, LDL receptor, and cholesterol-7α-hydroxylase. However, the frequent cholesterol intake down-regulated the mRNA level of hepatic LDL receptor. In contrast, the frequent cholesterol intake up-regulated the mRNA levels of intestinal Niemann−Pick C1-like 1 (NPC1L1), acyl coenzyme A:cholesterol acyltransferase 2 (ACAT2), and microsomal triacylglycerol transport protein (MTP). It was concluded that the cholesterol intake frequency-induced elevation in plasma TC was associated with greater cholesterol absorption, possibly mediated by up-regulation of NPC1L1, ACAT2, and MTP.

KEYWORDS: Cholesterol; consumption frequency; SREBP; HMG CoA reductase; CYP7A1; liver X receptor; LDL receptor; NPC1L1; ABCG5; ACAT2; MTP

INTRODUCTION

Cholesterol has been long blamed as a culprit in the incidence of coronary heart disease (CHD). Epidemiological studies have demonstrated that elevated levels of plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) are the major risk factors, whereas high concentrations of plasma high-density lipoprotein cholesterol (HDL-C) and a low ratio of TC to HDL-C are protective ones against CHD (*1*). It is known that eating frequency influences blood lipoprotein profile and glucose metabolism (*2*). Plasma lipids including cholesterol itself have been shown to increase when animals are switched from a nibbling diet to a gorging diet pattern (*2*,*3*). In humans, regular meal frequency has beneficial effects on fasting lipid and postprandial insulin profile (*4*−*6*). However, results from other studies have been inconclusive (*7*, *8*).

Regulation of cholesterol absorption plays an important role in maintaining the whole body cholesterol homeostasis (*9*). It is estimated that about 1200−1700 mg of cholesterol enters the lumen of the small intestine with 300−500 mg coming from the diet and the remainder deriving from bile each day (*10*, *11*). Cholesterol absorption starts with the transporter Niemann−Pick C1-like 1 (NPC1L1), which transports cholesterol from the lumen into enterocytes. Intestinal acyl-CoA:cholesterol acyltransferase 2 (ACAT2) then converts cholesterol to cholesteryl ester (CE) (**Figure 1**) followed by the microsomal triacylglycerol transport protein (MTP), which packs CE into chylomicrons (CM). Finally, CM is transferred into blood through the lymphatic system (*12*, *13*). The ATP-binding cassette transporter subfamily G members 5 and 8 (ABCG5/8) return the unabsorbed free cholesterol and most phytosterols left in the enterocytes to the lumen for excretion (*14*, *15*).

Cholesterol homeostasis is maintained at the transcriptional level by sterol regulatory element-binding protein 2 (SREBP) and liver X receptor α (LXRα) in a coordinated manner (*16*). SREBP governs the transcription of 3-hydroxy-3-methylglutaryl reductase (HMG-CoA-R) and low-density lipoprotein receptor (LDL-R), with HMG-CoA-R acting as the rate-limiting enzyme in cholesterol synthesis and LDL-R being responsible for the removal of LDL-C from the circulation (**Figure 1**). LXRα regulates the transcription of a gene encoding cholesterol-7α-hydroxylase (CYP7A1), which converts cholesterol to bile acids for elimination (*16*).

Despite extensive investigation on the link between dietary cholesterol and plasma lipoprotein profile, no study to date has investigated how cholesterol intake frequency interacts with the

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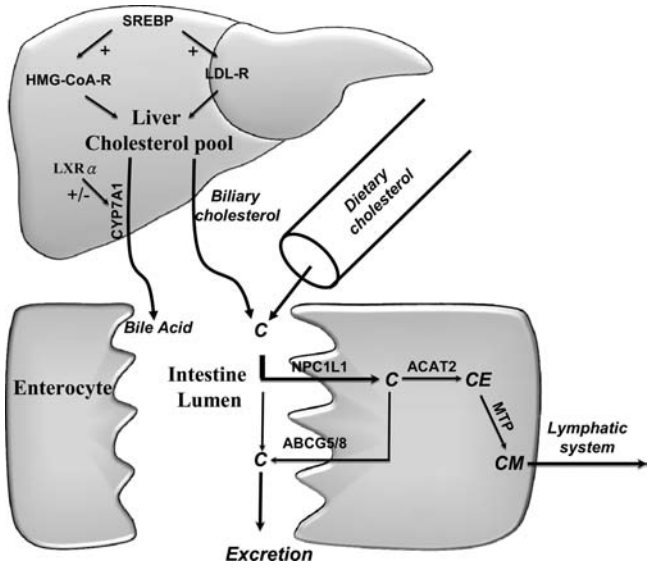

Figure 1. Cholesterol (C) absorption and excretion of bile acids. Cholesterol in the lumen is derived from two sources, namely, diet and bile. Sterol regulatory element binding protein 2 (SREBP) regulates the gene expression of LDL receptor (LDL-R) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA-R), whereas liver X receptor α (LXRα) governs the gene expression of cholesterol 7α-hydroxylase (CYP7A1). Cholesterol 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 triacylglycerols (MTP) into chylomicrons (CM) and transferred into blood through the lymphatic system. ATP binding cassette transporter subfamily G members 5 and 8 (ABCG5/8) return minor amounts of unabsorbed cholesterol to the lumen of the intestine for excretion. +, up-regulation; −, down-regulation.

gene of these transporters, proteins, and enzymes involved in regulation of cholesterol homeostasis. The present study was therefore the first time to (i) examine the effect of cholesterol intake frequency on the gene expression of intestinal NPC1L1, ABCG 5 and 8, ACAT2, and MTP and to (ii) characterize how cholesterol intake frequency interacts with SREBP, LXRα, HMG-CoA-R, LDL-R, and CYP7A1 in hamsters.

MATERIALS AND METHODS

Diets. All diets were prepared as previously described (*17*). A cholesterol-free diet was prepared by mixing the following ingredients (g/kg of diet): cornstarch, 508; casein, 242; lard, 50; sucrose, 119; mineral mix, 40; vitamin mix, 20; DL-methionine, 1. A 0.1% cholesterol diet was prepared by adding 0.1% cholesterol by weight into the cholesterol-free diet. The powdered diets were mixed with a gelatin solution (20 g/L) in a ratio of 200 g of diet/L. Once the gelatin had set, the diets were cut into pieces of approximately 10 g cubes and stored frozen at −20 °C.

Animals. Male adult Golden Syrian hamsters (*Mesocricetus auratus*; $n = 33$, 117 ± 9 g) were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. All experiments were conducted following approval and in accordance with the guidelines set by the Animal Experimental Ethical Committee, The Chinese University of Hong Kong.

Hamsters were randomly divided into three groups ($n = 11$), namely CD, C-3, and C-1 groups. They were housed in wire-bottom cages at 23 °C in an animal room with a 12 h light−dark cycle. All of the hamsters were adapted to gavage feeding three times of 0.5 mL of 3% starch solution per day for 1 week before the experiments. They were allowed free access to food and water. During the following 6 weeks, the CD group was maintained on a 0.1% cholesterol diet throughout the entire study (high cholesterol intake frequency). It was found that CD hamsters consumed about 9 g of food daily, which contained 9 mg of cholesterol. C-3 hamsters, that is, the regular cholesterol intake frequency group, were daily gavage-administered 3 mg of cholesterol in 0.5 mL of 3% starch solution three times at 9:00 a.m., 4:00 pm, and 10:00 pm, respectively, which were the three peak times of food consumption by hamsters. C-1 hamsters with a low cholesterol intake frequency were daily gavage-administered 9 mg of cholesterol in 0.5 mL of 3% starch solution one time; the other two times, the C-1 group was orally given 0.5 mL of 3% starch solution containing no cholesterol. Similarly, the CD group was also gavage-administered three times 0.5 mL of 3% starch solution containing no cholesterol. Regardless of the modes of cholesterol given, all three groups had 9 mg of cholesterol intake. During the entire period of study, food was given daily; any uneaten food was discarded, and the amount of food consumed was measured. Body weights were recorded, and total feces were collected weekly. Blood (1 mL) was obtained from the retro-orbital sinus into a heparinized capillary tube at the beginning and end of weeks 2, 4, and 6 following food deprivation for 14 h and light anesthesia, using a mixture of ketamine, xylazine, and saline (v/v/v, 4:1:5). The blood was centrifuged at 1000*g* for 10 min, and the plasma was collected and stored at −20 °C until analysis. Following the last collection of blood sample at week 6, all of the hamsters were kept for 3 days to allow for recovery and then euthanized by carbon dioxide suffocation after 14 h of food deprivation. Liver, heart, kidney, and adipose tissues (perirenal and epididymal pads) were removed, washed in saline, and weighed. The first 10 cm of duodenum was discarded, and the following 30 cm of the small intestine was kept, with the first 15 cm for mRNA analysis and the second 15 cm for measuring the ACAT enzyme activity and immunoblot analysis. All tissue samples were flash frozen in liquid nitrogen and stored at −80 °C until analysis.

Determination of Plasma Lipoproteins. Plasma TC and total triacylglycerols (TG) were quantified using commercial enzymatic kits from Thermo (Waltham, MA) and Stanbio Laboratories (Boerne, TX), respectively. For measurement of plasma HDL-C, LDL-C and very low-density lipoprotein cholesterol (VLDL-C) were first precipitated with phosphotungstic acid and magnesium chloride using a commercial kit (Stanbio Laboratories). Non-HDL-C was calculated by deducting HDL-C from TC.

Measurement of Organ Cholesterol. Cholesterol concentrations in the liver, heart, kidney, and adipose tissues were quantified using stigmastanol as an internal standard (*18*, *19*). In brief, total lipids were extracted into chloroform/methanol (2:1, v/v) and were then saponified in 90% ethanol containing 1.0 N NaOH. The cholesterol in the nonsaponified substances was converted to its trimethylsilyl (TMS) ether derivatives using a commercial TMS reagent (Sigma-Sil-A, Sigma, St. Louis, MO). The analysis of cholesterol TMS ether derivative was performed in a fused silica capillary column (SAC-5, 30 m × 0.25 mm, i.d.; Supelco, Inc., Bellefonte, PA) in a Shimadzu GC-14 B GLC equipped with a flame ionization detector as previously described.

Determination of Fecal Neutral and Acidic Sterols. Individual fecal neutral and acidic sterols were quantified using stigmasterol and hyodeoxycholic acid as internal standards, respectively (*19*). The freeze-dried fecal samples were ground and well mixed followed by saponification in 90% ethanol containing 1.0 N NaOH. The total neutral sterols were extracted into cyclohexane and were then converted to their corresponding TMS ether derivatives for GLC analysis. For quantification of acidic sterols, to the remaining aqueous layer was added 10 M NaOH followed by heating at 120 °C for 3 h. After cooling, distilled water and 3 N HCl solution were added followed by extraction of diethyl ether twice. The diethyl ether layers were then pooled and mixed with 2 mL of methanol, 2 mL of dimethoxypropane, and 40 μL of concentrated HCl (12 N). After standing overnight at room temperature, the solvents were dried down and the acidic sterols were similarly converted to their TMS ether derivatives at 60 °C for GLC analysis.

Western Blotting Analysis of Liver SREBP, LDL-R, HMG-CoA-R, LXRα, and CYP7A1. Western blotting analyses were carried out as we previously described (*20*, *21*). Total liver proteins were extracted into a homogenizing buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 0.2 M sucrose, and Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany) followed by centrifugation at 13000*g*. The supernatant was centrifuged at 126000*g*, and the pellet was resuspended in the same homogenizing buffer. The proteins were separated on a 7% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF)

Table 1. Quantitative Real-Time PCR Primers Used To Measure the Intestinal RNA Levels of Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH), Cholesterol-7α-hydroxylase (CYP7A1), 3-Hydroxy-3-methylglutaryl-CoA Reductase (HMG-CoA-R), LDL Receptor (LDL-R), Sterol Regulatory Element Binding Protein 2 (SREBP), Niemann−Pick C1-like 1 (NPC1L1), ATP Binding Cassette Transporter Subfamily G Members 5 and 8 (ABCG5/8), Acyl Coenzyme A:Cholesterol Acyltransferase 2 (ACAT2), and Microsomal Triacylglycerols (MTP)

gene	NCBI accession no. or ref	orward primer 5′→3′	reverse primer 5′→3′
TaqMan			
GAPDH	DQ403055	GAACATCATCCCTGCATCCA	CCAGTGAGCTTCCCGTTCA
CYP7A1	L04690	GGTAGTGTGCTGTTGTATATGGGTTA	ACAGCCCAGGTATGGAATCAAC
HMG-CoA-R	X00494	CGAAGGGTTTGCAGTGATAAAGGA	GCCATAGTCACATGAAGCTTCTGT A
LDL-R	M94387	GCCGGGACTGGTCAG ATG	ACAGCCACCATTGTTGTCCA
SREBP-2	U12330	GGACTTGGTCATGGGAACAGATG	TGTAATCAATGGCCTTCCTCAGAAC
SYBR Green			
NPC1L1	DQ897680	CCTGACCTTTATAGAACTCACCACAGA	GGGCCAAAATGCTCGTCAT
ABCG5	ref *22*	TGATTGGCAGCTATAATTTTGGG	GTTGGGCTGCGATGGAAA
ABCG8	ref *22*	TGCTGGCCATCATAGGGAG	TCCTGATTTCATCTTGCCACC
ACAT2	ref *23*	CCGAGATGCTTCGATTTGGA	GTGCGGTAGTAGTTGGAGAAGGA
MTP	ref *24*	GTCAGGAAGCTGTGTCAGAATG	CTCCTTTTTCTCTGGCTTTTCA
18S	M33069	TAAGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC

membranes (Millipore, Billerica, MA) using a semidry transfer system. Membranes were then blocked in 5% nonfat milk Tris-buffered saline with Tween-20 for 1 h and overnight at 4 °C in the same solution containing anti-LDL-R antibody (Calbiochem, EMD Chemicals, Inc., San Diego, CA), anti-HMG-CoA-R (Upstate USA Inc., Lake Placid, NY), anti-CYP7A1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-SREBP-2 antibody (Santa Cruz Biotechnology), or anti-LXRα antibody (Santa Cruz Biotechnology) . The membrane was then incubated for 1 h at 4 °C in diluted horseradish peroxidase-linked goat anti-rabbit IgG (Santa Cruz Biotechnology), donkey anti-goat IgG (Santa Cruz Biotechnology), and goat anti-mouse IgG (Santa Cruz Biotechnology). Then, membranes were developed with ECL enhanced chemiluminescence agent (Santa Cruz Biotechnology) and subjected to autoradiography on SuperRX medical X-ray film (Fuji, Tokyo, Japan). Densitometry was quantified using the Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA). Data on abundance of SREBP-2, LDLR, HMG-CoA-R, LXRα, and CYP7A1 were normalized with β-actin (Santa Cruz Biotechnology).

Real-Time PCR Analysis of mRNA for Liver SREBP-2, LDL-R, HMG-CoA R, LXR, and CYP7A1 and Small Intestine NPC1L1, ABCG5, ABCG8, ACAT2, and MTP. Total mRNA levels in the liver SREBP-2, LDL-R, HMG-CoA R, LXR, and CYP7A1 were quantified as previously described (*17*). In brief, total RNA was extracted and converted to cDNA (cDNA) using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Reverse transcription was carried out in a thermocycler (Gene Amp PCR system 9700, Applied Biosystems), with the program set as initiation for 10 min at 25 °C, followed by incubation at 50 °C for 90 min and at 85 °C for additional 5 min. The cDNA synthesized was stored at −20 °C. Total small intestinal mRNA was similarly extracted and converted to its cDNA.

Real-time PCR analysis was carried out on a Fast Real-time PCR System 7500 (Applied Biosystems) for both the hepatic and small intestinal genes. Primers and TaqMan probes were used for real-time PCR for the liver GAPDH, CYP7A1, HMG-CoA-R, LDL-R, SREBP, and LXRα in hamsters, whereas for the small intestinal NPC1L1, ABCG5, ABCG8, ACAT2, MTP, and 18S, SYBR green was used as a fluorophore (**Table 1**). The reaction mixture was subjected to thermal cycling under the following conditions: heating to 95 °C in 20 s, followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. Data were analyzed using the Sequence Detection Software version 1.3.1.21 (Applied Biosystems). Gene expressions were calculated according to the comparative threshold cycle (C_T) method (Applied Biosystems).

Measurement of Intestinal ACAT Activity and Western Blotting Analysis of Intestinal MTP. ACAT activity was quantified as we previously described (*25*). In brief, the intestinal microsome was obtained, and the esterification reaction was initiated by adding an assay reagent consisting of [^{14}C]oleoyl-coenzyme A, nonradioactive oleoyl-coenzyme A, and fatty acid-free bovine serum albumin. The reaction was stopped by adding a chloroform/methanol mixture (2:1, v/v) and saline. After the addition of [^{3}H]cholesterol oleate, the reaction mixture was centrifuged and the lower organic layer was collected followed by the addition of cholesteryl oleate. Cholesterol and cholesteryl ester were separated on a thin-layer silica gel plate (Merck, Rahway, NJ) in hexane/ethyl acetate/acetic acid (80:20:1, v/v). The band corresponding to cholesterol oleate was cut off and transferred into a scintillation vial followed by the addition of OptiPhase HiSafe 2 scintillation fluid (Perkin-Elmer). Radioactivity was then measured in an LS 6500 scintillation counter (Beckman), and the data were calculated on the basis of ^{3}H recovery.

Total intestinal microsome proteins were separated on a 7% SDS-PAGE gel and transferred to PVDF membranes (Millipore, Billerica, MA) using a semidry transfer system. Membranes were then blocked in 5% nonfat milk, Tris-buffered saline with Tween-20 for 1 h and overnight at 4 °C in the same solution containing antimicrosomal triacylglycerol transfer protein (MTP) polyclonal antibody (BD Biosciences, Palo Alto, CA). The membrane was then incubated for 1 h at 4 °C in diluted horseradish peroxidase-linked goat anti-mouse IgG (Santa Cruz Biotechnology). Then, membranes were developed with ECL enhanced chemiluminescence agent (Santa Cruz Biotechnology) and subjected to autoradiography on SuperRX medical X-ray film (Fuji). Densitometry was quantified using the Bio-Rad Quantity One software (Bio-Rad Laboratories). Data on abundance of MTP were normalized with β-actin (Santa Cruz Biotechnology).

Statistics. Data were expressed as mean ± standard deviation (SD). The group means were statistically analyzed using one-way analysis of variance (ANOVA) and post hoc LSD test on SigmaStat Advisory Statistical Software (SigmaStat version 14.0, SPSS Inc., Chicago, IL). Significance was defined as a p value of < 0.05.

RESULTS

Food Intake; Body and Organ Weights. No significant differences in the final body weights were seen among the three groups. Similarly, there were no significant differences in food intakes among the three groups. The ratios of liver, heart, epididymal fat, and perirenal fat to the final body weight were similar among the three groups.

Serum TC, HDL-C, TG, and Non-HDL-C/HDL-C. There was no difference in plasma lipoprotein profiles among the three groups at week 0 (**Table 2**). At the end of week 2, plasma TG demonstrated a decreasing trend among the CD, C-3, and C-1 groups (**Table 2**). At the end of week 4, plasma TC, non-HDL-C, TC/HDL-C ratio, and TG decreased with the decreasing cholesterol intake frequency among CD (high), C-3 (regular), and C-1 (low) groups. Similarly, the decreasing cholesterol intake frequency lowered plasma TC, HDL-C, non-HDL-C, TC/HDL-C ratio, and TG at the end of week 6. To be specific, plasma TC levels were 243.52, 210.18, and 196.91 mg/dL, whereas plasma TG levels were 173.58, 141.83, and 85.38 mg/dL in groups CD, C-3, and C-1, respectively.

Organ Cholesterol. No differences in heart cholesterol content were seen among the three groups. In contrast, cholesterol

Table 2. Changes in Plasma Total Cholesterol (TC), Triacylglycerols (TG), High-Density Lipoprotein Cholesterol (HDL-C), and Non-HDL Cholesterol (Non-HDL-C) in Hamsters Fed the 0.1% Cholesterol Diet (CD), the Cholesterol-free Diet with Gavage Administration of 3 mg of Cholesterol Three Times per Day (C-3), or the Cholesterol-free Diet with Gavage Administration of 9 mg of Cholesterol One Time per Day (C-1)[a]

	CD	C-3	C-1	p_{trend}
week 0				
TC (mg/dL)	167.05 ± 24.24	166.48 ± 20.40	166.36 ± 20.47	1.00
HDL-C (mg/dL)	111.38 ± 10.36	112.30 ± 9.63	113.80 ± 28.38	0.78
non-HDL-C (mg/dL)	55.67 ± 26.3	54.18 ± 15.70	52.38 ± 16.84	0.89
TC/HDL-C	1.49 ± 0.09	1.48 ± 0.14	1.46 ± 0.14	0.78
TG (mg/dL)	109.42 ± 41.05	116.53 ± 31.88	103.80 ± 28.38	0.69
week 2				
TC (mg/dL)	198.07 ± 21.61	195.23 ± 24.76	187.56 ± 17.98	0.51
HDL-C (mg/dL)	113.77 ± 14.35	106.59 ± 8.98	112.02 ± 4.87	0.24
non-HDL-C(mg/dL)	84.30 ± 15.13	88.63 ± 21.21	75.53 ± 16.39	0.23
TC/HDL-C	1.75 ± 0.16 ab	1.83 ± 0.19 a	1.67 ± 0.14 b	0.11
TG (mg/dL)	154.79 ± 71.08 a	122.91 ± 49.00 ab	88.55 ± 18.48 b	0.02
week 4				
TC (mg/dL)	233.01 ± 23.20 a	211.14 ± 25.71 b	196.14 ± 11.19 b	<0.01
HDL-C (mg/dL)	125.52 ± 8.46 a	119.13 ± 7.54 b	118.89 ± 4.81 b	0.06
non-HDL-C (mg/dL)	107.49 ± 17.92 a	92.01 ± 23.66 ab	77.25 ± 11.50 b	<0.01
TC/HDL-C	1.86 ± 0.12 a	1.77 ± 0.20 ab	1.65 ± 0.10 b	0.01
TG (mg/dL)	148.18 ± 56.70 a	120.08 ± 48.31 a	87.05 ± 20.79 b	0.01
week 6				
TC (mg/dL)	243.52 ± 21.48 a	210.18 ± 19.30 b	196.91 ± 14.85 b	<0.01
HDL-C (mg/dL)	123.42 ± 8.98 a	109.78 ± 6.74 b	107.14 ± 6.19 b	<0.01
non-HDL-C (mg/dL)	120.10 ± 16.24 a	100.40 ± 14.86 b	89.77 ± 11.55 b	<0.01
TC/HDL-C	1.97 ± 0.12 a	1.91 ± 0.12 ab	1.84 ± 0.10 b	0.03
TG (mg/dL)	173.58 ± 76.16 a	141.83 ± 68.57 a	85.38 ± 21.29 b	<0.01
organ cholesterol				
liver (mg/g)	57.72 ± 6.10 a	38.92 ± 4.95 b	32.08 ± 4.79 c	<0.01
heart (mg/g)	1.66 ± 0.07	1.62 ± 0.11	1.65 ± 0.13	0.60
kidney (mg/g)	4.02 ± 0.18 a	3.87 ± 0.28 ab	3.70 ± 0.18 b	0.01
adipose tissue (mg/g)	1.27 ± 0.14 a	1.11 ± 0.21 b	1.01 ± 0.09 b	<0.01

[a] Data are expressed as mean ± SD, $n = 11$. p_{trend} detects the statistical significance across the intake frequency. Means in a row with different letters differ significantly between two groups, $p < 0.05$.

Table 3. Changes (Milligrams) in Daily Total Cholesterol Intake, Fecal Excretion of Total Neutral Sterols, Total Acidic Sterols, and Cholesterol Balance in Hamsters Fed the 0.1% Cholesterol Diet (CD), the Cholesterol-free Diet with Gavage Administration of 3 mg of Cholesterol Three Times per Day (C-3), or the Cholesterol-free Diet with Gavage Administration of 9 mg of Cholesterol One Time per Day (C-1)[a]

	CD	C-3	C-1	p_{trend}
week 2				
cholesterol intake (mg)	8.54 ± 0.45 b	9.00 ± 0.00 a	9.00 ± 0.00 a	0.36
neutral sterol (mg)	1.17 ± 0.20 c	4.09 ± 0.69 b	4.99 ± 0.49 a	<0.01
acidic sterol (mg)	1.79 ± 0.39	2.38 ± 1.31	1.92 ± 0.51	0.53
cholesterol retained (mg)	5.58 ± 0.56 a	2.53 ± 1.23 b	2.09 ± 0.86 b	<0.01
retained/intake (%)	65.18 ± 3.64 a	28.07 ± 13.65 b	23.23 ± 9.60 b	<0.01
week 6				
cholesterol intake (mg)	9.64 ± 0.44 a	9.00 ± 0.00 b	9.00 ± 0.00 b	<0.01
neutral sterol (mg)	1.29 ± 0.32 b	4.23 ± 0.89 a	4.98 ± 0.66 a	<0.01
acidic sterol (mg)	1.63 ± 0.48	1.48 ± 0.55	1.54 ± 0.24	0.89
cholesterol retained (mg)	6.72 ± 0.65 a	3.29 ± 1.23 b	2.48 ± 0.68 b	<0.01
retained/intake (%)	69.83 ± 7.14 a	36.51 ± 13.64 b	27.59 ± 7.58 b	<0.01

[a] Data are expressed as mean ± SD ($n = 11$). p_{trend} detects the statistical significance across the intake frequency. Means in a row with different letters differ significantly between two groups, $p < 0.05$.

content in the liver, kidney, and adipose tissues demonstrated a decreasing trend in the CD, C-3, and C-1 groups.

Cholesterol Balance and Excretion of Fecal Neutral and Acidic Sterols. Total intake of cholesterol was compared with its excretion in neutral and acidic sterols (**Table 3**). Net cholesterol equivalent retained was calculated by difference between the intake and excretion of both neutral and acidic sterols. It was found that the net cholesterol retention of the CD group was greatest followed by that in C-3 and C-1 groups. The apparent cholesterol absorption was calculated by the equation [(cholesterol intake − excretion of neutral and acidic sterols)]/cholesterol intake]. It was clear that the increasing cholesterol intake frequency (C-1 → CD) increased the apparent cholesterol absorption.

Immunoblot and mRNA Analyses of Liver SREBP, LDL-R, HMG-CoA-R, LXRα, and CYP7A1. The Western blot and real-time PCR analyses demonstrated that frequency of cholesterol intake had no significant effect on hepatic SREBP, HMG-CoA-R, LXRα, and CYP7A1 in hamsters (**Figure 2**). Although the immunoblot analysis showed that frequency of cholesterol

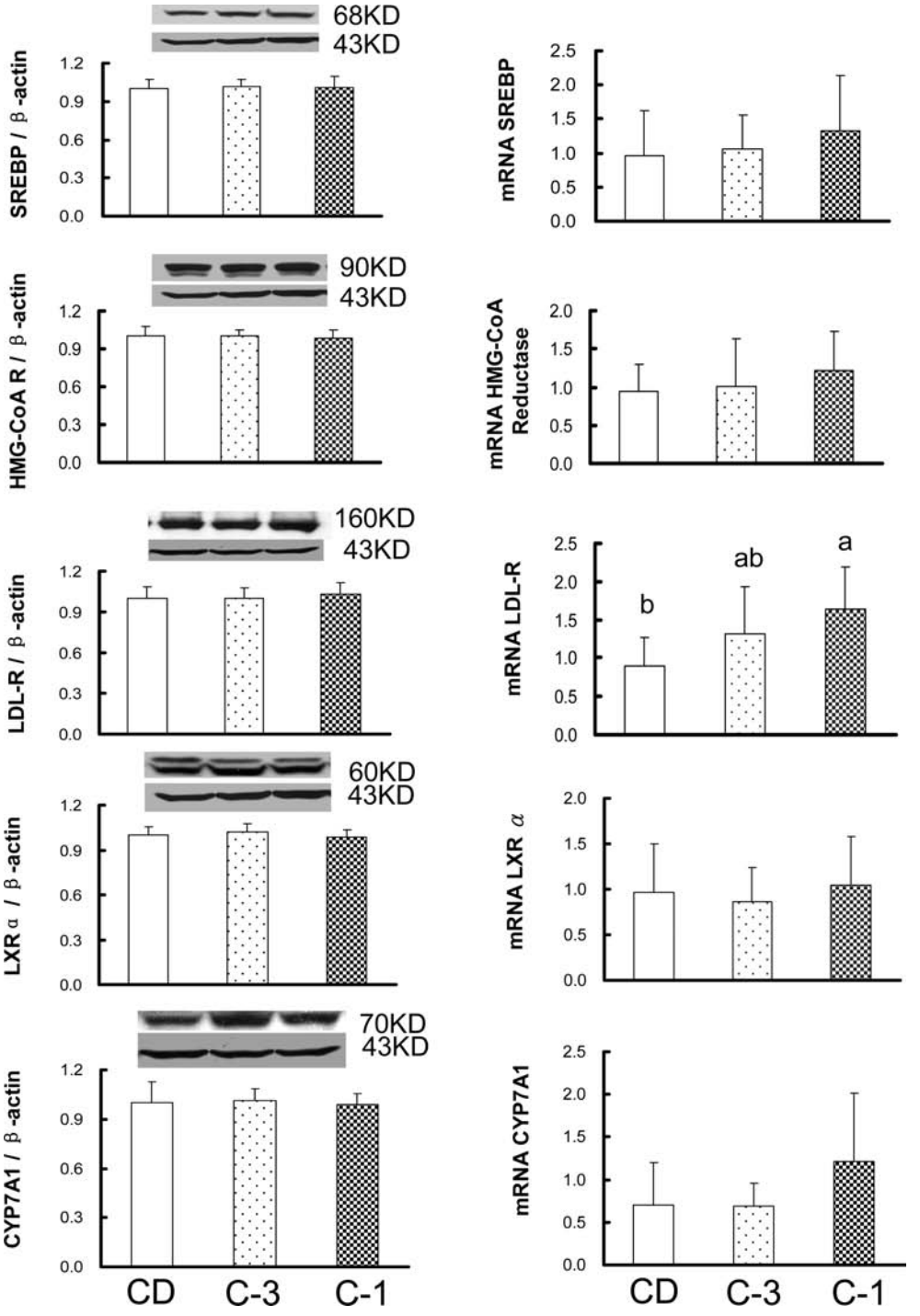

Figure 2. Relative immunoreactive mass and mRNA of hepatic sterol regulatory element-binding protein-2 (SREBP), 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA-R), LDL receptor (LDL-R), liver X receptor (LXRα), and cholesterol-7α-hydroxylase (CYP7A1) in hamsters fed the 0.1% cholesterol diet (CD), the cholesterol-free diet with gavage administration of 3 mg of cholesterol three times per day (C-3), or the cholesterol-free diet with gavage administration of 9 mg of cholesterol one time per day (C-1).

intake had no effect on the protein mass of LDL-R, real-time PCR analysis demonstrated that increasing cholesterol intake frequency could down-regulate the expression of mRNA LDL-R (**Figure 2**)

mRNA Analyses of Intestinal ABCG5, ABCG8, NPC1L1, MTP, and ACAT2. No clear trend was seen on these transporters, enzymes, and proteins involved in cholesterol absorption between the C-3 and C-1 groups. However, real-time PCR analyses demonstrated that the cholesterol in diet (CD group) was associated with greater expression of mRNA ABCG5, NPC1L1, ACAT2, and MTP compared with that in the C-3 and C-1 groups (**Figure 3**).

Intestinal ACAT Activity and Immunoblot Analysis of Intestinal MTP. With the increasing frequency of cholesterol intake (C-1 → CD), the intestinal ACAT activity increased (**Figure 4**). The C-1 group had its intestinal ACAT activity reduced by 58.4 and 52.8%, respectively, compared with the CD and C-3 groups. The immunoblot analysis indicated that the protein mass of intestinal MTP of the C-1 group was significantly lower than that of both the CD and C-3 groups.

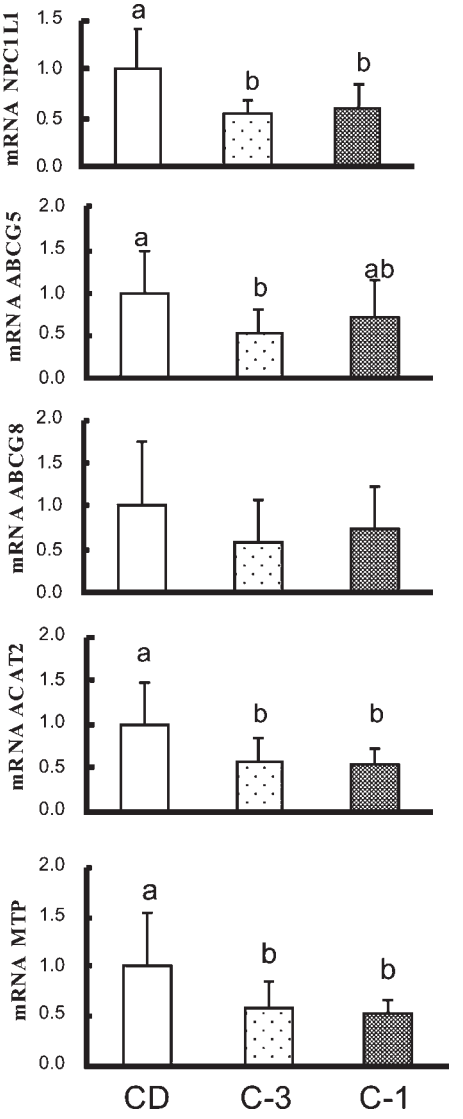

Figure 3. Relative mRNA of intestinal Niemann−Pick C1-like 1 (NPC1L1), ATP-binding cassette (ABC) transporter subfamily G members 5 and 8 (ABCG5/8), acyl-CoA:cholesterol acyltransferase 2 (ACAT2), and microsomal triglyceride transfer protein (MTP) in hamsters fed the 0.1% cholesterol diet (CD), the cholesterol-free diet with gavage administration of 3 mg of cholesterol three times per day (C-3), or the cholesterol-free diet with gavage administration of 9 mg of cholesterol one time per day (C-1).

DISCUSSION

Cholesterol intake frequency increases plasma total cholesterol for a given amount in diet. In view of the difficulty in studying the effect of cholesterol intake frequency on plasma TC and LDL-C levels in humans, hamsters as a hypercholesterolemia model were chosen because their cholesterol metabolism is similar or close to that in humans (*21*, *26*). In the present study, hamsters were given daily 9 mg of cholesterol either in the diet (CD) or by a gavage administration of 3 × 3 mg (C-3) and 1 × 9 mg (C-1). Results demonstrated clearly that there was an increasing trend in concentrations of plasma TC, non-HDL-C (LDL-C + VLDL-C), TC/HDL-C ratio, and TG in association with the cholesterol intake frequency (**Table 2**). It is the first demonstration that the increasing cholesterol intake frequency increased the apparent cholesterol absorption (**Table 3**). This effect was reflected in the observation that the C-1 group hamsters excreted most fecal neutral sterols followed by C-3 and CD hamsters. Attention has been paid to not only total amount of cholesterol intake but also intake modes if the data can be extrapolated to humans.

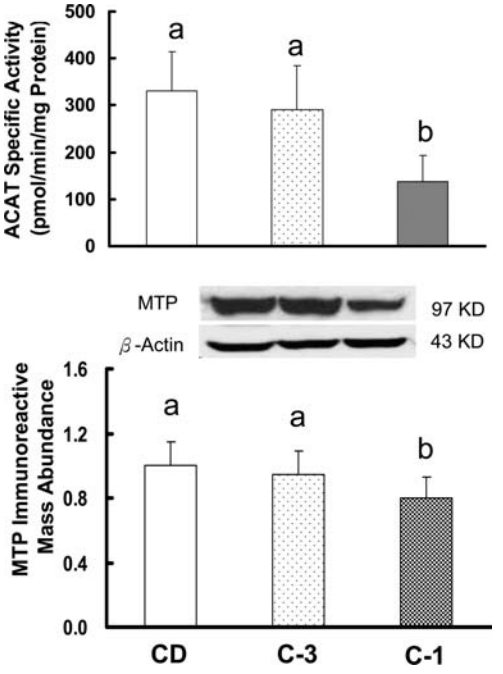

Figure 4. Intestinal acyl coenzyme A:cholesterol acyltransferase (ACAT) activity and immunoblot analyses of intestinal MTP in hamsters fed the 0.1% cholesterol diet (CD), the cholesterol-free diet with gavage administration of 3 mg of cholesterol three times per day (C-3), or the cholesterol-free diet with gavage administration of 9 mg of cholesterol one time per day (C-1).

The underlying mechanism by which the frequent cholesterol intake increased the cholesterol absorption remains poorly understood. It should be pointed out that the gavaged hamsters were given cholesterol without the presence of any other lipids. It was possible cholesterol was much better transported to the endothelial cells when it was mixed with dietary lipids. The cholesterol absorption is governed by two types of transporters, namely, NPC1L1 and ABCG 5/8 (*12*, *14*). The former is an influx transporter responsible for channeling the cholesterol from the intestine lumen into enterocytes, whereas the latter are the efflux transporters responsible for shuttling some unesterified cholesterol back to the lumen for excretion. We hypothesize that efficient cholesterol absorption associated with frequent cholesterol intake for a given amount of total dietary cholesterol is mediated by up-regulation of intestinal NLC1L1. In fact, real-time PCR analyses demonstrated that mRNA NPC1L1 in the CD group was up-regulated compared with those in C-3 and C-1 hamsters. However, no difference in expression of NPC1L1 was seen between groups C-3 and C-1, suggesting that other factor(s) may also be involved in the regulation of cholesterol absorption. In this regard, the two enzymes, namely, ACAT2 and MTP, are also essential in the regulation of cholesterol absorption, with the former promoting intracellular sterol esterification in the enterocytes, whereas MTP is responsible for the chylomicron assembly (*27*). Results showed the CD group had greater mRNA levels of ACAT2 and MTP compared with C-3 and C-1 hamsters. Although no differences in mRNA ACAT2 and MTP were seen between the C-3 and C-1 groups, the intestinal ACAT activity in the C-1 group was reduced by 52.8% compared with that in C-3 hamsters, suggesting that the frequent cholesterol intake increased intestinal ACAT activity without necessarily up-regulating its mRNA level. Similarly, MTP had greater protein masses in

CD and C-3 groups than in the C-1 group. The present study clearly demonstrates that cholesterol intake frequency affected cholesterol absorption, possibly mediated by regulation of these sterol transporters and enzymes.

It remains unknown how the cholesterol intake frequency interacts with the gene expressions of receptors, proteins, and enzymes involved in cholesterol elimination pathway. Excessive cholesterol is eliminated via the following two mechanisms. First, cholesterol can incorporate into bile fluid and be eliminated as fecal neutral sterols (**Figure 1**). Second, cholesterol is converted to bile acids and eliminated as fecal bile acids. Our data demonstrate that cholesterol intake frequency did not affect the excretion of bile acids and had no effect on the gene expression of SREBP, LXRα, HMG-CoA-R, and CYP7A1 (**Table 3**). However, CD hamsters accumulated most cholesterol in the liver followed by C-3 and C-1 groups in decreasing order (**Table 3**) with down-regulation of LDL-R (**Figure 2**). The present study confirms the phenomenon of the inverse relationship between hepatic cholesterol and LDL-R number. The lower hepatic cholesterol in the C-1 group led to an up-regulation of LDL-R (*28*).

Humans consume 200−600 mg of cholesterol/day. However, mode of cholesterol intake varies with individuals, with some people taking a large amount of cholesterol in one meal with the other two meals containing little cholesterol and others taking cholesterol more or less similarly among the three meals. Although the data in the present study could not be directly extrapolated to what happen in humans, the present study was the first time to demonstrate that for a given amount of dietary cholesterol, increasing consumption frequency elevated plasma TC, non-HDL-C, TG, and hepatic cholesterol levels. Elevation of plasma TC is most likely mediated by up-regulation of NPC1L1, ACAT2, and MTP gene expression.

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