RESEARCH ARTICLE

Dietary calcium decreases plasma cholesterol by down-regulation of intestinal Niemann–Pick C1 like 1 and microsomal triacylglycerol transport protein and up-regulation of CYP7A1 and ABCG 5/8 in hamsters

Ka Ying Ma[1], Nan Yang[1], Rui Jiao[1], Cheng Peng[1], Lei Guan[1], Yu Huang[2] and Zhen-Yu Chen[1]

[1] Food & Nutritional Sciences Programme of Department of Biochemistry, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, P. R. China
[2] School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, P. R. China

Received: April 1, 2010
Revised: June 16, 2010
Accepted: June 19, 2010

Scope: It has been shown that calcium supplementation favorably modifies plasma lipoprotein profile in postmenopausal women. The present study investigated the interaction of dietary calcium with genes of transporters, receptors and enzymes involved in cholesterol metabolism.

Methods and results: Forty-eight ovariectomized hamsters were fed one of the four diets containing 0, 2, 6 and 8 g calcium per kg. Plasma total cholesterol (TC), triacylglycerols (TG), and non-high density lipoprotein cholesterol were dose-dependently decreased, whereas high-density lipoprotein cholesterol (HDL-C) was dose-dependently increased with the increasing dietary calcium levels. Dietary calcium had no effect on protein mass of hepatic sterol regulatory element binding protein-2 (SREBP), liver X receptor-alpha (LXR), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), LDL receptor (LDLR) and cholesterol-7α-hydroxylase (CYP7A1). However, dietary calcium up-regulated the mRNA levels of hepatic CYP7A1 and intestinal ATP binding cassette transporters (ABCG5/8) whereas it down-regulated the intestinal Niemann-Pick C1 like 1 (NPC1L1) and microsomal triacylglycerol transport protein (MTP). In addition, dietary calcium increased the activity of intestinal acyl coenzyme A: cholesterol acyltransferase 2, while it decreased plasma cholesteryl ester transport protein (CETP).

Conclusion: Beneficial modification of lipoprotein profile by dietary calcium was mediated by sequestering bile acid absorption and enhancing excretion of fecal cholesterol, *via* up-regulation of mRNA CYP7A1 and intestinal ABCG 5/8 with down-regulation of mRNA NPC1L1 and MTP.

Keywords:
ABCG 5/8 / CYP7A1 / LDL cholesterol / Niemann–Pick C1 like 1 / Total cholesterol

1 Introduction

Plasma total cholesterol (TC) and LDL cholesterol (LDL-C) correlate positively, whereas HDL cholesterol (HDL-C) correlates inversely with coronary heart disease. Calcium supplementation has been shown to decrease plasma TC in rabbit [1], hamster [2], rat [3, 4], pig [5] and humans [6]. It has been also demonstrated that calcium supplementation improves the lipoprotein profile in hyperlipidemia patients [7]. Particularly in menopausal women, calcium supplementation not only decreases LDL-C but also increases HDL-C level [8]. It appears that dietary calcium is more effective in reducing plasma TC in women than that in men

Correspondence: Professor Zhen-Yu Chen, Department of Biochemistry, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, P. R. China
E-mail: zhenyuchen@cuhk.edu.hk
Fax: +852-2603-7246

Abbreviations: ACAT2, acyl coenzyme A: cholesterol acyltransferase 2; **CE,** cholesteryl ester; **CETP,** cholesteryl ester transport protein; **HDL-C,** HDL cholesterol; **HMGR,** 3-hydroxy-3-methylglutaryl-CoA reductase; **LDL-C,** LDL cholesterol; **LDLR,** LDL receptor; **LXR,** liver X receptor; **MTP,** microsomal triacylglycerol transport protein; **NPC1L1,** Niemann–Pick C1 like 1; **SREBP,** sterol regulatory element-binding protein; **TC,** total cholesterol; **TG,** triacylglycerol; **TMS,** trimethylsilyl-ether

[6, 8]. It has been proposed that the beneficial effect of calcium supplementation on the lipoprotein profile is mediated by its enhanced activity on fecal excretion of bile acids [9].

Intestinal absorption of cholesterol is a transporter-facilitated process (Fig. 1). Niemann–Pick C1 like 1 (NPC1L1) localizes to the brush border membrane of absorptive enterocytes in the small intestine and is responsible for the uptake of cholesterol [10]. Intestinal acyl coenzyme A: cholesterol acyltransferase 2 (ACAT2) esterifies cholesterol to form cholesteryl ester (CE). Microsomal triacylglycerol transport protein (MTP) assembles CE into chylomicrons, which is subsequently transferred into blood through the lymphatic system. ACAT can differentiate cholesterol from phytosterols and prefer the former as a substrate [11]. ATP-binding cassette transporters (ABCG5 and ABCG8) return some cholesterol and most phytosterols to the lumen of the intestine for excretion (Fig. 1).

LDL carries most cholesterol in circulation in humans while in rodents HDL does most [12]. Although lipoprotein metabolism differs across species, elimination of cholesterol is in general regulated at the transcriptional level by sterol regulatory element-binding protein 2 (SREBP) and liver X receptor (LXR) in a coordinated manner [13, 14]. SREBP governs the transcription of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and LDL receptor (LDLR), with HMGR acting as the rate-limiting enzyme in cholesterol synthesis, whereas LDLR being responsible for the removal of LDL-C from the circulation (Fig. 1). LXR regulates the transcription of a gene encoding cholesterol-7α-hydroxylase (CYP7A1), which is a rate-limiting enzyme in conversion of cholesterol to bile acids in the liver and elimination in the bile fluid [14].

Despite extensive research on the link between dietary calcium and plasma lipoprotein profile, information on the interaction of dietary calcium with the genes of these transporters, receptors and enzymes involved in cholesterol metabolism *in vivo* is lacking. In view of the pronounced hypocholesterolemic activity of calcium supplementation in postmenopausal women [6, 8], this study adopted ovariectomised hamsters as a model to (i) examine the effect of dietary calcium on the gene expression of intestinal NPC1L1, ABCG 5 and 8, ACAT and MTP; and (ii) characterize how dietary calcium interact with hepatic SREBP, LXR, HMGR, LDLR and CYP7A1.

2 Materials and methods

2.1 Diets

All diet ingredients were purchased from Harlan Teklad (Madison, WI, USA) except for lard, which was obtained from the local market (the same batch for all the experiments). DL-methionine, cholesterol and calcium dibasic phosphate were purchased from Sigma Chemical (St. Louis, MO, USA). The calcium-deficient diet (Ca-0) was prepared by mixing the following ingredients (g/kg diet): cornstarch, 508; casein, 242; lard, 50; sucrose, 119; calcium-deficient mineral mix, 40; vitamin mix, 20; DL-methionine, 1; cholesterol, 1. The other three experimental diets were prepared by adding 5 g of $CaHPO_4$ (equivalent to 2 g Ca/kg diet; Ca-2), 15 g of $CaHPO_4$ (equivalent to 6 g Ca/kg diet; Ca-6) and 20 g of $CaHPO_4$ (equivalent to 8 g Ca/kg diet; Ca-8), respectively, into Ca-0 diet. In view of about 6 g Ca/kg in rodent chow diet, Ca-0, Ca-2, Ca-6 and Ca-8 diets represented the amount of calcium deficiency, inadequacy, adequacy and sufficiency, respectively. The powered diets were mixed with a gelatin solution (20 g/L) in a ratio of 200 g diet *per* liter. Once the gelatin has set, the diets were cut into pieces of approximately 10 g cubes and stored frozen at

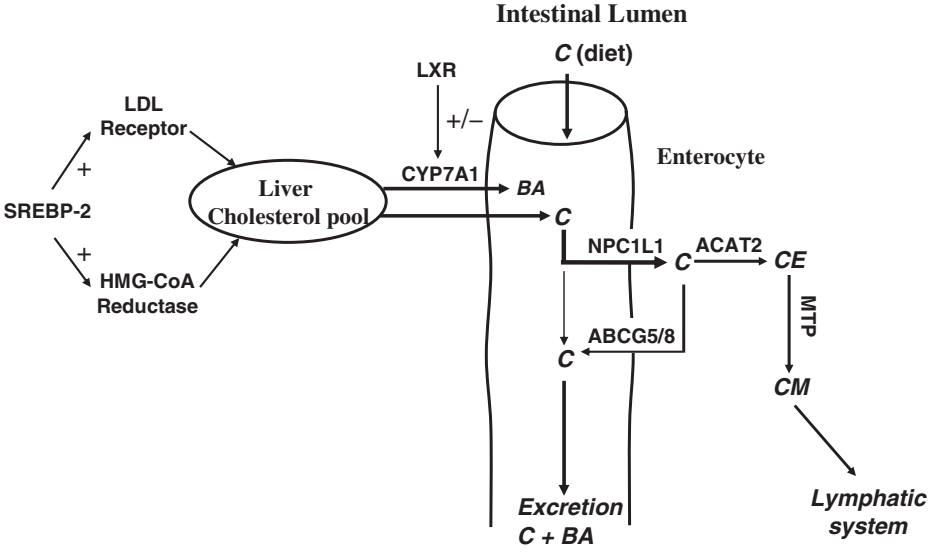

Figure 1. Cholesterol (**C**) absorption and excretion of bile acid (**BA**) and **C**. SREBP2 regulates the gene expression of LDLR and HMGR, whereas LXR aplpha governs the gene expression of cholesterol 7α-hydroxylase (CYP7A1). **C** is transported into enterocytes *via* intestinal NPC1L1. Intestinal ACAT2 esterifies cholesterol to form CE, which is packed with microsomal triacylglycerols (MTP) into chylomicrons and transferred into blood through the lymphatic system. ATP-binding cassette transporters (ABCG5 and ABCG8) return minor amount of cholesterol to the lumen of the intestine for excretion. +, up-regulation. –, down-regulation.

−20°C. In this study, the atherogenic diets containing 0.1% cholesterol were chosen in order to investigate the interaction of dietary calcium with absorption of dietary cholesterol.

2.2 Hamsters

Forty-eight ovariectomized Golden Syrian hamsters (*Mesocricetus auratus*; 120–130 g) were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. Experiments were conducted following the approval and in accordance with the guidelines set by the Animal Experimental Ethical Committee, The Chinese University of Hong Kong. Ovariectomized hamsters were randomly divided into four groups ($n = 12$) and housed (two *per* cage) in wire-bottomed cages at 23°C in an animal room with 12-h light–dark cycle. All hamsters were acclimatized to the Ca-8 diet for a period of 2 wk and subsequently maintained on their respective diets for additional 6 wk. Respective diets and water were available *ad libitum*, with any uneaten food being weighed and replaced with fresh food daily. Body weights were measured and the fecal samples *per* cage were collected weekly. Blood (1 mL) was obtained from the retro-orbital sinus into a heparinized capillary tube at the end of weeks 0, 3 and 6 following food deprivation for 14 h over night and light anaesthesia, using a mixture of ketamine, xylazine and saline 1:1:5, (4:1:5; v/v/v). After the blood was centrifuged at $1000 \times g$ for 10 min, the plasma was collected and stored at −20°C until analysis. Following the last blood sample collection at week 6, all the hamsters were killed by carbon dioxide suffocation. The livers 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.

2.3 Quantification of calcium in plasma, diet and feces

Calcium in plasma, diet and feces was measured according to the AOAC Official Method 968.08 (AOAC International, 1995). In brief, the sample (2.0 g) was weighed into a porcelain crucible and placed into a furnace at 550°C for 4 h. The carbon-free ash residues were cooled at room temperature and dissolved in 10 mL of 3 M HCl solution followed by being boiled for 10 min. The solution was cool and diluted to a volume of 100 mL. The measurement solution was then prepared by twofold dilution with addition of 1% lanthanum oxide (AAS grade, Sigma) to reduce phosphate interference. The standard solution of calcium (Sigma) was subjected to the same procedure. The solution was quantified in a Varian Spectra-800 atomic absorption spectrophotometer. Instrumental conditions were set at a pump flow rate, 3.0 mL/min; wavelength, 422.7 nm; lamp current, 10 mA; slit width, 0.5 nm; and air/C_2H_2 flow ratio, 3 to 1. Plasma calcium concentration was measured using a commercial kit (Sigma).

2.4 Measurement of plasma lipoproteins

Plasma TC and triacylglycerols (TG) were quantified using the enzymatic kits from Infinity (Waltham, MA, USA.) and Stanbio Laboratories (Boerne, TX, USA), respectively [15]. To measure plasma HDL-C, LDL and very LDL were first precipitated with phosphotungstic acid and magnesium chloride using a commercial kit (Stanbio Laboratories). HDL-C in the supernatant was determined similarly as it was for TC. Non-HDL-C was calculated by deducting HDL-C from TC.

2.5 Quantification of liver cholesterol

Cholesterol concentration in the liver was determined using a method as we described previously [15, 16]. Briefly, stigmastanol as an internal standard was added into the liver sample. Methanol-chloroform mixture (2:1, v/v) were used to extract total liver lipids. The chloroform–methanol layer was saved and evaporated to dryness under a gentle stream of nitrogen gas. The liver lipids were then mildly saponified and the cholesterol was converted into its trimethylsilyl-ether (TMS) derivative before the GC analysis.

2.6 Quantification of fecal neutral and acidic sterols

Neutral and acidic sterols in the feces were analyzed as we described previously [15, 16]. In brief, stigmasterol and hyodeoxycholic acid were added into the fecal samples as internal standards for quantification of fecal neutral and acidic sterols, respectively. After being mildly hydrolyzed, the fecal neutral sterols were extracted into cyclohexane and were converted into their TMS derivatives. The acid sterols in the bottom aqueous layer were saponified, extracted and converted into their TMS derivatives. Both the analyses of individual neutral and acidic sterol TMS derivatives were performed in a fused silica capillary column (SAC-5, 30 m × 0.25 mm, id; Supelco, Bellefonte, PA, USA) using a Shimadzu GC-14 B Gas-Liquid Chromatograph equipped with a flame ionization detector (Kyoto, Japan). Stigmasterol and hyodeoxycholic acid added in the samples were used to quantify each neutral and acidic sterols, respectively. The total neutral sterols were the sum of cholesterol, coprostanol, coprostanone, dihydrocholesterol, campesterol, β-sistosterol amd stigmastanol, whereas the total acid sterols were the sum of lithocholic, deoxycholic, chenodeoxycholic, cholic and ursodeoxycholic acids.

2.7 Measurement of intestinal ACAT activity

Intestinal ACAT activity was measured according to the method we described previously [17]. Briefly, the intestinal microsome was prepared and incubated with cholesterol. The reaction was initiated by adding an assay reagent consisting of [^{14}C] oleoyl-Coenzyme A, non-radioactive oleoyl-Coenzyme A and fatty acid-free bovine serum albumin. The reaction was stopped by adding chloroform: methanol mixture (2:1, v/v) and saline. After addition of [^{3}H] cholesterol oleate, the reaction mixture was centrifuged and the lower organic layer was collected followed by addition of cholesteryl oleate. Cholesterol and CE were separated on a thin-layer silica gel plate (Merck, NJ, USA) in hexane:ethyl acetate:acetic acid (80:20:1, v/v/v). The band corresponding to cholesterol oleate was cut off and transferred into a scintillation vial followed by addition of OptiPhase HiSafe 2 scintillation fluid (Perkin-Elmer). Radioactivity was then measured in a LS 6500 scintillation counter (Beckman) and the data were calculated based on [^{3}H] recovery.

2.8 Quantification of plasma cholesteryl ester transport protein

Plasma CETP concentration was measured using a commercial kit (ALPCO, Salem, NH, USA). In brief, plasma (10 μL) was diluted to 1:80 with citrate buffer (pH 5.5) and added to the tested wells coated with anti-CETP MoAb followed by incubation for 2 h. CETP in the plasma was captured by the antibody. After washing to remove all the unbound materials, HRP-labeled anti-CETP MoAb was added. After incubation for 1 h and subsequent washing, the substrate solution was added followed by addition of the stop reagent. The intensity of color that developed was read by a microplate reader. The absorbance was proportional to the concentration of CETP in the plasma.

2.9 Determination of plasma 25-hydroxyvitamin D

Plasma 25-hydroxyvitamin D was considered to be the most reliable measure of overall vitamin status. It was quantified using an enzyme immunoassay kit from the Immunodiagnostic Systems (Boldon Business Park, UK)

2.10 Western blotting analyses of intestinal MTP and liver SREBP, LDL-R, HMGR, LXR and CYP7A1

Western blotting analyses were carried out as we described previously [15]. Briefly, the frozen liver was homogenized, the extract was centrifuged,and the supernatant was collected and considered the ''total protein'' [18]. After the total protein was centrifuged at 35 000 rpm, the supernatant was removed and the pellet (membrane and nuclear protein) was saved followed by re-suspension into the same homogenizing buffer. The protein concentration was determined using a protein concentration assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The target proteins were separated on a 7% SDS-PAGE gel and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) using a semi-dry transfer system. Membranes were then blocked in 5% non-fat milk Tris-buffered saline with Tween-20 for 1 h and overnight at 4°C in the same solution containing anti-LDL-R antibody (Santa Cruz Biotechnology, CA, USA), anti-HMGR (Upstate USA, Lake Placid, NY, USA), anti-CYP7A1 (Santa Cruz Biotechnology), anti-LXR, or anti-SREBP antibody (Santa Cruz Biotechnology) [18]. The membrane was then incubated for 1 h at 4°C in diluted horseradish peroxidase-linked Goat anti-rabbit IgG, donkey anti-rabbit IgG or goat anti-mouse IgG. The membranes were developed with ECL enhanced chemiluminescence agent and subjected to autoradiography on SuperRX medical X-ray film. Densitometry was quantified using the BioRad Quantity one® software. Data on abundance of SREBP, LDLR, HMG R, LXR and CYP7A1 were normalized with β-actin [15].

2.11 Real-time PCR analyses of mRNA of liver SREBP, LDLR, HMGR, LXR, CYP7A1, ABCG5, ABCG8, MTP and small intestine NPC1L1, ABCG5, ABCG8, ACAT and MTP

Both liver and intestine samples were pulverized with liquid nitrogen in a mortar, and total liver mRNA was extracted and isolated using Tizol® Reagent (Invitrogen, Carlsbad, CA, USA). Briefly, total RNA from liver and intestine was converted to cDNA using a high-capacity cDNA reverse transcription kit, respectively (Applied Biosystems, Foster City, CA, USA). Reverse transcription was carried out in a thermocycler (Gene Amp® PCR system 9700, Applied Biosystems), with 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.

Real-time PCR analysis was carried out on a Fast Real-time PCR System 7500 (Applied Biosystems). Primers and TaqMan® probes were used for real-time PCR analysis of liver GAPDH, CYP7A1, HMGR, LDL-R, SREBP-2 and LXR [15]. For intestine NPC1L1, ABCG5, ABCG8, ACAT2, MTP and 18S, SYBR green was used as fluorophore [19–21] (Table 1). The reaction mixture was subject to thermal cycling under the following conditions: heating up 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).

Table 1. Quantitative real-time PCR primers used to measure hamster intestine RNA levels

Gene	Forward primer 5′ 3′	Reverse primer 5′ 3′
GAPDH	GAACATCATCCCTGCATCCA	CCAGTGAGCTTCCCGTTCA
CYP7A1	GGTAGTGTGCTGTTGTATATGGGTTA	ACAGCCCAGGTATGGAATCAAC
HMG-CoA R	CGAAGGGTTTGCAGTGATAAAGGA	GCCATAGTCACATGAAGCTTCTGT A
LDL-R	GCCGGGACTGGTCAG ATG	ACAGCCACCATTGTTGTCCA
SREBP-2	GGACTTGGTCATGGGAACAGATG	TGTAATCAATGGCCTTCCTCAGAAC
NPC1L1	CCTGACCTTTATAGAACTCACCACAGA	GGGCCAAAATGCTCGTCAT
ABCG5	TGATTGGCAGCTATAATTTTGGG	GTTGGGCTGCGATGGAAA
ABCG8	TGCTGGCCATCATAGGGAG	TCCTGATTTCATCTTGCCACC
ACAT2	CCGAGATGCTTCGATTTGGA	GTGCGGTAGTAGTTGGAGAAGGA
MTP	GTCAGGAAGCTGTGTCAGAATG	CTCCTTTTTCTCTGGCTTTTCA
18S	TAAGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC

2.12 Statistics

Data were expressed as mean ± SD. The group means were statistically analyzed using two-way analysis of variance (ANOVA) and *post hoc* LSD test on SigmaStat Advisory Statistical Software (SigmaStat version 14.0, SPSS, Chicago, IL, USA). Significance was defined as *p*-value less than 0.05.

3 Results

3.1 Food intake, body and organ weight

No significant differences in the final body weight were observed among the four ovariectomized groups. Similarly, there were no significant differences in food intakes among the four groups (data not shown). The weights of kidney, heart, liver and adipose tissues (epididymal and perirenal pads) were similar among the four groups.

3.2 Quantification of calcium in plasma, diet and feces

Plasma calcium concentrations increased with the increasing dietary calcium level with Ca-8 group being 13.8 ± 1.3 mg/dL (range: 12.3–16.4 mg/dL) followed by Ca-6 group being 12.5 ± 0.8 mg/dL (range: 11.3–14.0 mg/dL), Ca-2 group being 11.2 ± 0.7 mg/dL (range: 10.3–12.1 mg/dL) and Ca-0 group being 10.1 ± 0.5 mg/dL (range: 9.5–10.4 mg/dL). The actual calcium concentration in diets of Ca-0, Ca-2, Ca-6 and Ca-8 groups was 0.4 ± 0.1, 2.3 ± 0.2, 6.2 ± 0.3 and 8.3 ± 0.4 g/kg, respectively. Fecal calcium excretion demonstrated a dose-dependent increase with the increasing dietary calcium (Ca-0, 5.8 ± 2.8 mg/g; Ca-2, 36.6 ± 11.3 mg/g; Ca-6, 87.1 ± 6.5 mg/g and Ca-8, 125.2 ± 12.8 mg/g).

3.3 Plasma TC, HDL-C, TG and non-HDL-C/HDL-C in ovariectomized hamsters

Four groups of ovariectomized hamsters had similar levels of plasma TC, HDL-C and TG at the beginning of the experiment (week 0). At the end of week 3, plasma TC, non-HDL-C and TC:HDL-C were dose-dependently decreased, whereas HDL-C was dose-dependently increased with the increasing dietary calcium levels (Table 2). When the experiment reached the end of week 6, the calcium-induced elevation of HDL-C and reduction in plasma TC, non-HDL-C and TC:HDL-C became more significant (Table 2). Dietary calcium levels had no effect on plasma TG at the end of week 3. However, plasma TG demonstrated a dose-dependent decrease with the increasing dietary calcium at the end of week 6 (Table 2).

3.4 Liver cholesterol

Hepatic cholesterol demonstrated a dose-dependent decrease pattern with the increasing dietary calcium levels (Table 2). Compared with that in Ca-0 group, liver cholesterol was reduced by 2% in Ca-2, by 17% in Ca-6 and 21% in Ca-8 group.

3.5 Plasma 25-hydroxyvitamin D

Vitamin D promotes the intestinal absorption of calcium. However, no difference in plasma 25-hydroxyvitamin D was seen among the four ovariectomized groups fed Ca-0, Ca-2, Ca-6 and Ca-8 diets (Table 2).

3.6 Fecal excretion of neutral and acidic sterols

Total fecal outputs in weeks 1, 3 and 6 were pooled and their concentrations of neutral sterols and acidic sterols were analyzed and calculated as milligrams *per* day. To simplify the

Table 2. Changes in hepatic cholesterol and plasma TC, total TG, HDL-C, non-HDL-C, TC:HDL-C, vitamin D, compesterol and lathosterol in ovariectomized hamsters fed the Ca-deficient diet (Ca-0) and three experimental diets supplemented with 2 g (Ca-2), 6 g (Ca-6) and 8 g (Ca-8) Ca *per* kg diet[a)], respectively

	Ca-0	Ca-2	Ca-6	Ca-8	*p*
Week 0					
TC (mg/dL)	210.4±34.07	211.5±51.5	210.5±33.3	211.9±36.3	1.00
TG (mg/dL)	123.1±49.2	134.7±68.4	124.1±44.3	116.69±58.3	0.89
HDL-C (mg/dL)	119.3±13.2	117.2±13.7	106.96±17.9	112.0±7.2	0.13
Non-HDL-C (mg/dL)	98.9±21.1	105.50±33.2	110.14±21.7	109.5±25.5	0.75
Non-HDL-C:HDL-C	0.82±0.21	0.86±0.22	1.05±0.24	0.96±0.22	0.109
TC:HDL-C	1.8±0.2	1.8±0.3	2.0±0.3	1.9±0.3	0.20
Week 3					
TC (mg/dL)	236.4±15.7a	229.4±19.2ab	221.4±19.7b	215.2±15.5b	0.04
TG (mg/dL)	132.5±35.3	121.4±37.0	142.3±20.5	133.5±17.9	0.33
HDL-C (mg/dL)	92.3±5.6b	94.5±3.4b	95.9±9.4b	109.0±15.90a	<0.01
Non-HDL-C (mg/dL)	143.2±14.5a	134.5±19.6ab	125.7±19.7b	104.1±22.5c	<0.01
Non-HDL-C:HDL-C	1.54±0.17a	1.42±0.21ab	1.33±0.26b	0.97±0.29c	<0.001
TC:HDL-C	2.5±0.2a	2.4±0.2ab	2.3±0.3b	2.0±0.3c	<0.01
Week 6					
TC (mg/dL)	261.1±39.3a	258.7±42.4a	242.7±33.6ab	222.2±11.5b	0.02
TG (mg/dL)	207.2±20.7a	179.8±27.8ab	163.6±30.0b	152.4±48.0c	<0.01
HDL-C (mg/dL)	104.5±4.2c	105.6±7.2c	129.4±13.8b	142.0±11.6a	<0.01
Non-HDL-C (mg/dL)	152.8±28.5c	123.5±85.5c	113.3±27.6b	81.5±16.5a	<0.01
Non-HDL-C:HDL-C	1.48±0.31a	1.40±0.30a	0.88±0.21b	0.58±0.16c	<0.001
TC:HDL-C	2.5±0.3c	2.4±0.3c	1.9±0.2b	1.6±0.2a	<0.01
25-Hydroxy D (μg/dL)	2.43±0.81	2.47±0.14	2.34±0.73	2.32±0.44	0.99
CETP (μg/mL)	0.59±0.04a	0.56±0.02a	0.54±0.06ab	0.50±0.040b	0.04
Liver cholesterol (mg/g)	41.5±8.3a	41.0±5.1a	34.4±4.0b	32.9±6.8b	0.04

a,b,c: in a row for a given week with different online letters differ significantly, $p<0.05$.
a) Values are means±SD, $n=10$.

Table 3. TC intake, fecal excretion of total neutral sterols, total acidic sterols (mg/hamster/day) and cholesterol balance in ovariectomized hamsters fed the Ca-deficient diet (Ca-0) and three experimental diets supplemented with 2 g (Ca-2), 6 g (Ca-6) and 8 g (Ca-8) Ca *per* kg diet[a)], respectively[a)], at week 6

	Ca-0	Ca-2	Ca-6	Ca-8	*p*
Cholesterol intake (CI)	11.55±0.82a	9.88±0.41b	10.60±0.82ab	10.71±0.94ab	0.10
Fecal neutral sterols (NS)					
Coprotanol	0.66±0.34b	1.44±0.50a	1.31±0.52ab	1.40±0.70a	0.41
Coprostanone	0.04±0.04	0.04±0.01	0.03±0.01	0.03±0.01	0.19
Cholesterol	0.34±0.23	0.31±0.15	0.64±0.31	0.47±0.43	0.44
Dihydrocholesterol	0.22±0.11	0.32±0.09	0.30±0.05	0.37±0.07	0.27
Campesterol	0.02±0.01	0.04±0.04	0.03±0.03	0.04±0.05	0.98
Total	1.28±0.41b	2.15±0.47a	2.31±0.64a	2.30±0.48a	0.07
Fecal acidic sterols (AS)					
Lithocholic	0.74±0.22	1.20±0.24	1.13±0.40	1.32±0.79	0.34
Deoxycholic	0.05±0.02b	0.05±0.01b	0.06±0.03b	0.14±0.08a	0.05
Chenodeoxycholic	0.33±0.07	0.28±0.08	0.17±0.10	0.47±0.35	0.35
Cholic acid	0.10±0.07b	0.26±0.07b	0.35±0.18ab	0.67±0.46a	0.04
Ursodeoxycholic	0.08±0.02b	0.07±0.02b	0.14±0.06ab	0.20±0.09a	0.03
Total	1.30±0.54b	1.87±0.29ab	1.85±0.62ab	2.81±1.70a	0.11
Apparent cholesterol retained (CR = CI−NS−AS)	9.13±1.31a	5.74±0.54b	5.90±1.52b	5.55±1.79b	0.04
Apparent cholesterol absorption (CR/CI)	78.98±8.99a	58.01±3.70b	55.23±10.86b	50.81±13.02b	0.04

a,b,c: at the same row with different online letters differ significantly, $p<0.05$.
a) Values are expressed as mean±SD, $n=10$.

presentation, only data in week 6 were shown (Table 3). Compared with those in Ca-0, total fecal neutral sterols were increased in the Ca-2, Ca-6 and Ca-8 groups. However, no significant differences were seen among the Ca-2, Ca-6 and Ca-8 groups. Coprostanol, cholesterol and dihydrocholesterol were the major neutral sterols in the feces. Although the effect of dietary calcium on fecal coprostanol was not dose-dependent, Ca-2 and Ca-8 groups had greater excretion than Ca-0 (Table 3).

It was apparent that the total acidic sterol excretion was increased in Ca-2, Ca-6 and Ca-8 groups compared with that in Ca-0 hamsters. Regarding the individual acidic sterols, deoxycholic, cholic and ursodeoxycholic acids were dose-dependently increased with the increasing dietary calcium (Table 3).

3.7 Cholesterol balance

Total intake of cholesterol was compared with the excretion of neutral and acidic sterols (Table 3). Cholesterol retention was calculated by difference between the intake and excretion of both neutral and acidic sterols. The apparent cholesterol absorption (cholesterol retention/cholesterol intake) demonstrated a dose-dependent decrease with the increasing dietary calcium levels.

3.8 Western blotting and mRNA analysis of SREBP-2, HMGR, LDLR, LXR and CYP7A1

Immunoreactive mass of liver SREBP-2, HMGR, LDLR, LXR and CYP7A1 was measured using Western blotting (Fig. 2). There were no significant differences in these proteins among the four ovariectomized groups. Similarly, no differences in mRNA abundance of SREBP, HMGR, LDLR and LXR in the liver were observed among the control and the four experimental groups (Fig. 3). However, hepatic mRNA CYP7A1 was up-regulated in a dose-dependent manner with the increasing dietary calcium ($p<0.05$).

3.9 Real-time PCR mRNA analysis of intestinal NPC1L1, ABCG5, ABCG8, ACAT2 and MTP

RT-PCR analysis demonstrated that calcium at 8 g/kg diet could up-regulate the production of mRNA ABCG5/8 (Fig. 4). In contrast, calcium at 8 g/kg diet down-regulated the mRNA level of MTP and NPC1L1, whereas it had no effect on the mRNA ACAT.

3.10 Real-time PCR mRNA analysis of hepatic ABCG5, ABCG8 and MTP

RT-PCR analysis demonstrated that calcium at 6 and 8 g/kg diet could up-regulate the production of mRNA ABCG5 with no effect on mRNA ABCG8 and MTP (Fig. 5).

3.11 Intestinal ACAT analysis

Dietary calcium at 8 g/kg suppressed intestinal ACAT activity (Fig. 6). However, calcium supplementation at level of 2 and 6 g/kg had no significant effect on the ACAT activity.

3.12 Plasma CETP concentration

Plasma CETP concentration in Ca-0, Ca-2, Ca-6 and Ca-8 groups was 0.59, 0.56, 0.54 and 0.50 μg/mL (Table 2), respectively, demonstrating a decreasing trend with the increasing dietary calcium ($p<0.05$).

4 Discussion

It remains unclear if dietary calcium-induced reduction in plasma-TC is dose-dependent. Daily calcium intake of about 1000 mg is generally recommended for adult population in humans. For rodents, purified diets, termed AIN-76A or AIN-93G and recommended by the American Institute of Nutrition, contain about 6 g calcium/kg diet [22]. Calcium levels of 0, 2, 6 and 8 g/kg diet adjusted in this study represented the amount of calcium deficiency, inadequacy, adequacy and sufficiency, respectively, in hamsters. Earlier studies have shown that calcium supplementation favorably modifies the lipoprotein profile in hamsters [2], rats [3, 4], pigs [5] and humans [7, 8], Jacqmain *et al.* [6] have examined the association between daily calcium intake and plasma lipoprotein profile in 235 men and 235 women, finding that daily calcium intake was negatively correlated with adiposity and plasma TC, LDL-C and TC:HDL-C ratio. A similar observation was made by Reid *et al.* [8], who investigated effect of calcium citrate supplementation (1 g/day) on fasting serum lipid concentrations in menopausal women, demonstrating that calcium supplement increased HDL-C level, decreased LDL-C:HDL-C ratio and caused beneficial changes in circulating lipids. In view of popularity of calcium supplementation among postmenopausal women [23], this study was the first to establish clearly that dietary calcium dose-dependently decreased plasma TC, non-HDL-C, TC/HDL-C and TG levels, whereas it dose-dependently increased plasma HDL-C level in ovariectomized hamsters, a model to study the cholesterol metabolism in postmenopausal women (Table 2). Results suggest that calcium supplementation beneficially models the circulating lipid profile in a dose-dependent manner.

In recent years, inhibition on plasma CETP as a strategy of raising plasma HDL-C has become popular [24–27]. Distribution of cholesterol among the lipoproteins is partially dependent on plasma CETP, which is a plasma

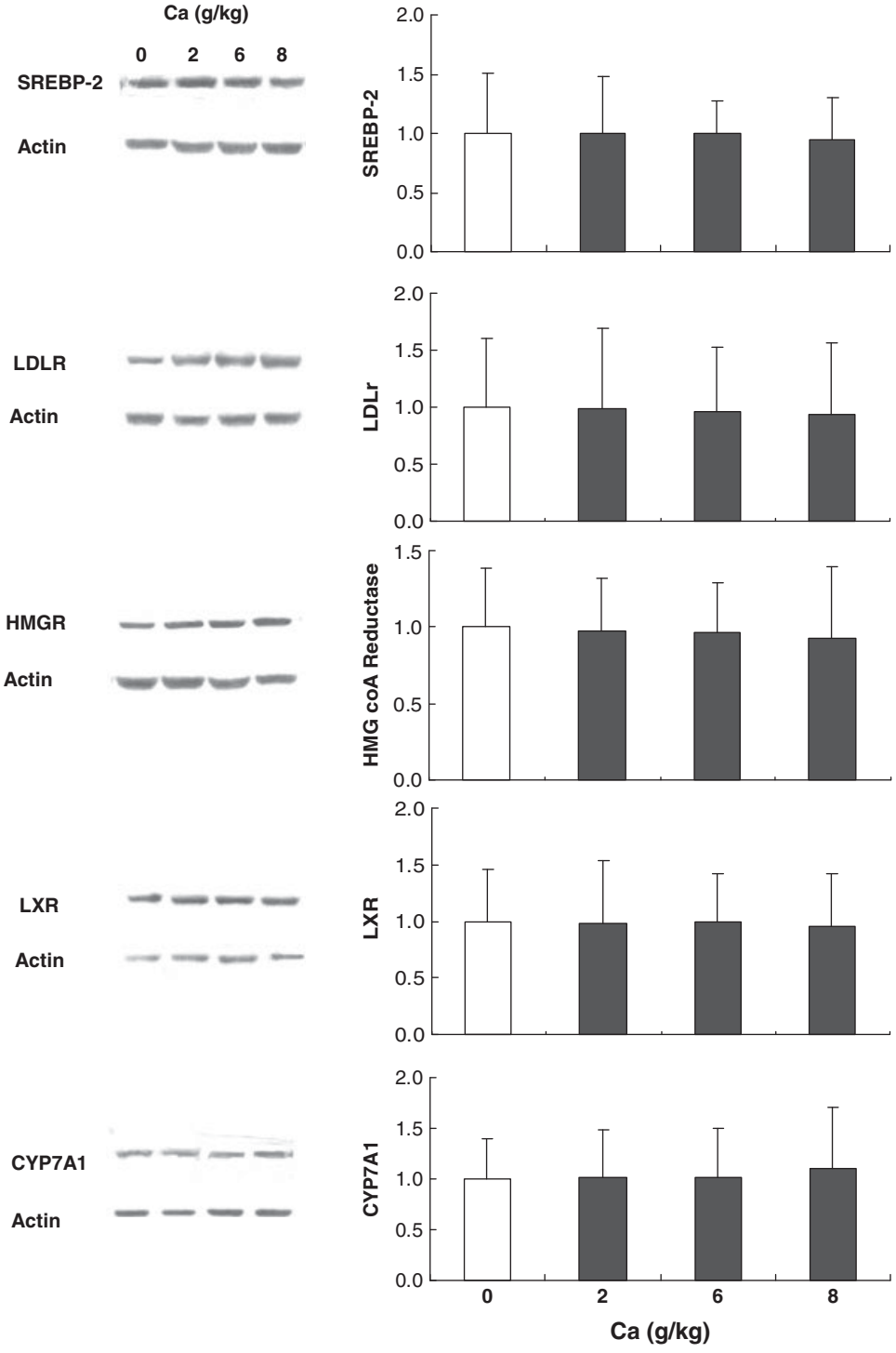

Figure 2. Effect of dietary calcium on the relative immunoreactive mass of hepatic SREBP2, LXR, HMGR, LDLR and cholesterol-7α-hydroxylase (CYP7A1) in hamsters fed the Ca-deficient diet (Ca-0) and three experimental diets supplemented with 2 g (Ca-2), 6 g (Ca-6) and 8 g (Ca-8) Ca *per* kg diet. Each lane was loaded with 80 μg proteins. Data are normalized with β-actin. Values are expressed as means $\pm$ SD ($n = 12$) with those for Ca-0 group being arbitrarily taken as one.

protein responsible for transferring CE from HDL to LDL with an exchange of the equivalent TG. This process is an essential part of the reversal cholesterol transport. Dietary calcium decreased the ratio of non-HDL-C (mainly LDL-C) to HDL-C, arousing our interest to study the effect of dietary calcium on plasma CETP concentration. Although the underlying mechanism remains unexplored, this study was the first time to demonstrate that the decrease in the ratio of non-HDL-C to HDL-C caused by the increasing dietary calcium was associated with the decreased CETP concentration (Table 2).

It remains unknown how dietary calcium interacts with the gene expressions of receptors, proteins and enzymes involved in cholesterol elimination pathway. Excessive

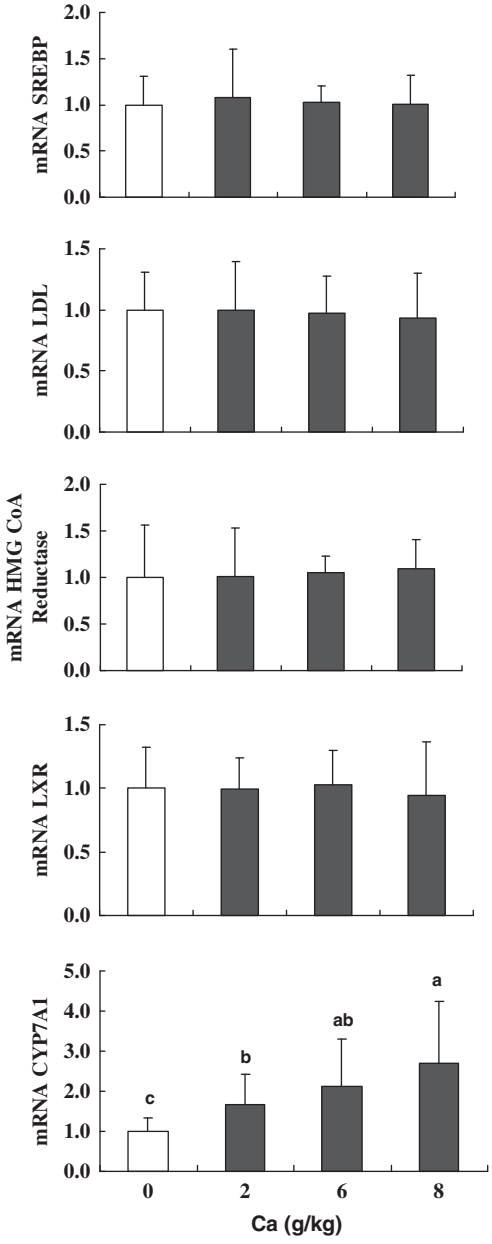

Figure 3. Effect of dietary calcium on mRNA levels of hepatic SREBP2, LXR, HMGR, LDLR and cholesterol-7α-hydroxylase (CYP7A1) in hamsters fed the Ca-deficient diet (Ca-0) and three experimental diets supplemented with 2 g (Ca-2), 6 g (Ca-6) and 8 g (Ca-8) Ca *per* kg diet. Data are normalized with GAPDH. Values are expressed as means ± SD ($n = 12$) with those for Ca-0 group being arbitrarily taken as one. a,b,c Means with different superscript letters differ significantly, $p < 0.05$.

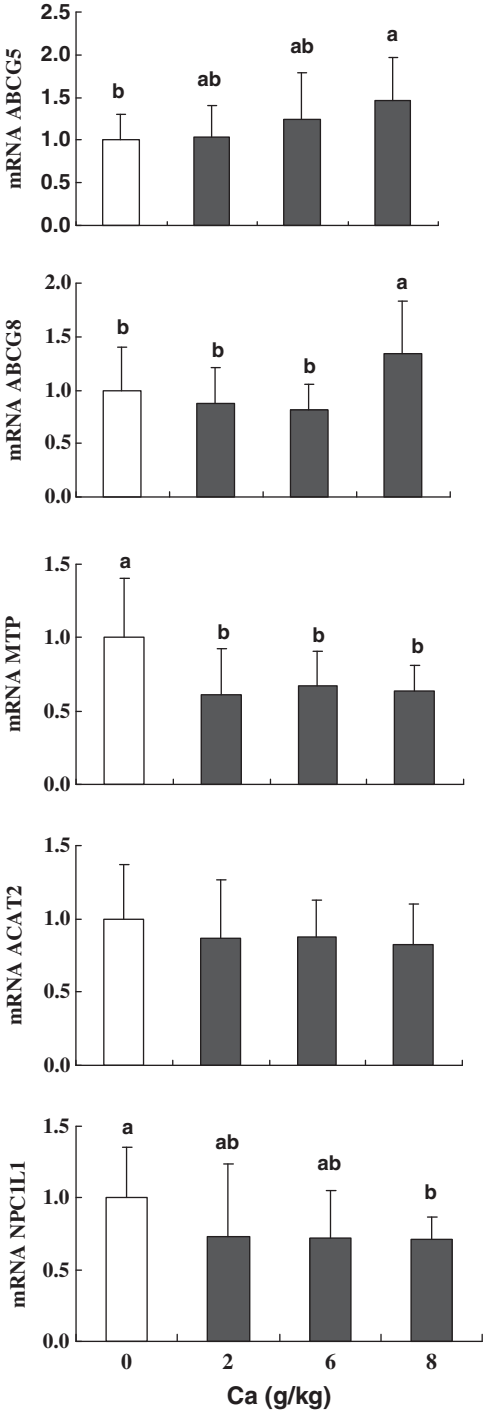

Figure 4. Effect of dietary calcium on mRNA levels of intestinal NPC1L1, ACAT2, MTP, ATP-binding cassette transporters (ABCG5 and ABCG8) in hamsters fed the Ca-deficient diet (Ca-0) and three experimental diets supplemented with 2 g (Ca-2), 6 g (Ca-6) and 8 g (Ca-8) Ca *per* kg diet. Data are normalized with cyclophilin. Values are expressed as means ± SD ($n = 12$) with those for Ca-0 group being arbitrarily taken as one. a,b Means with different superscript letters differ significantly, $p < 0.05$.

cholesterol is eliminated *via* the following two mechanisms. First, cholesterol can incorporate into bile fluid and eliminated as the fecal neutral sterols (Fig. 1). Second, cholesterol is converted to bile acids and eliminated as the fecal bile acids. It is known that calcium can bind bile acids to decrease their re-absorption and increase their excretion into

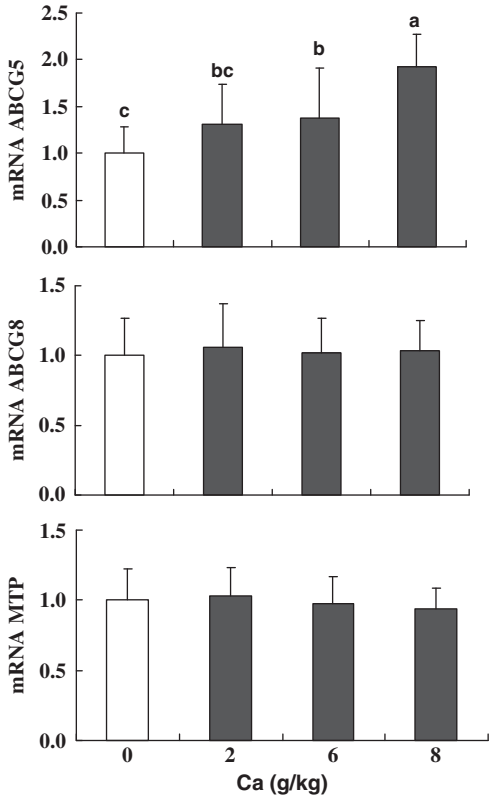

Figure 5. Effect of dietary calcium on mRNA levels of hepatic MTP, and ATP-binding cassette transporters (ABCG5 and ABCG8) in hamsters fed the Ca-deficient diet (Ca-0) and three experimental diets supplemented with 2 g (Ca-2), 6 g (Ca-6) and 8 g (Ca-8) Ca *per* kg diet. Data are normalized with cyclophilin. Values are expressed as means ± SD ($n = 12$) with those for Ca-0 group being arbitrarily taken as one. a,b,c Means with different superscript letters differ significantly, $p < 0.05$.

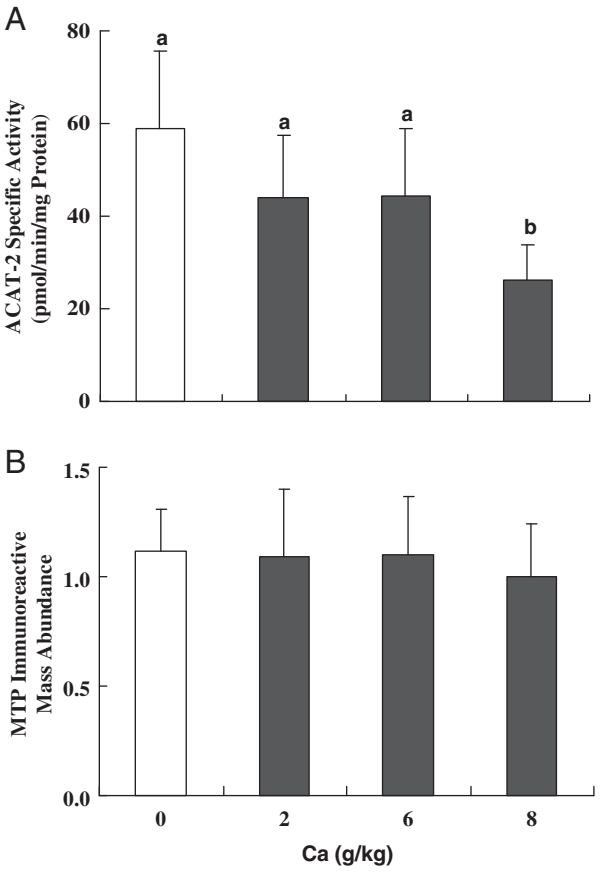

Figure 6. Effect of dietary calcium on intestinal enzymatic activity of ACAT2 and the relative immunoreactive mass of MTP in hamsters fed the Ca-deficient diet (Ca-0) and three experimental diets supplemented with 2 g (Ca-2), 6 g (Ca-6) and 8 g (Ca-8) Ca *per* kg diet. Data are normalized with cyclophilin. MTP data are normalized with β-actin. Values are expressed as means ± SD ($n = 12$). a,b Means with different superscript letters differ significantly, $p < 0.05$.

feces, thus leading to reduction in the liver and plasma cholesterol level [8, 9]. Our data showed that addition of calcium in diets increased fecal bile acids output (Table 3) and reduced the hepatic cholesterol concentrations (Table 2). In addition, we further investigated the effect of dietary calcium on SREBP, LDLR, HMGR, LXR and CYP7A1, finding there were no differences in the protein levels of SREBP-2, HMGR, LDLR, LXR and CYP7A1 among the four ovariectomized groups (Figs. 1 and 2). However, dietary calcium was able to up-regulate the mRNA CYP7A1 in a dose-dependent manner without affecting the mRNA levels of SREBP, HMGR, LDLR and LXR. We believe that driving force of dietary calcium to reduce plasma TC level starts in the intestine, where calcium binds bile acids to form a precipitate and increases the excretion of bile acids. This process is mediated by up-regulation of CYP7A1. To explore the underlying mechanism by which dietary calcium had no effect on the protein levels of SREBP, LDLR, HMGR, LXR and CYP7A1, the following explanations could be offered. Hamsters have a diurnal pattern in expression of HMGR with their activity in midnight being several times higher than that in midday [28]. Perhaps, the abundance in HMGR, SREBP, LDLR, LXR and CYP7A1 was already very low because hamsters in this study were sacrificed with an empty stomach, so that cholesterol catabolism rate was nil and no effect of dietary calcium in these proteins could be seen after the overnight fasting. In this regard, we are currently investigating the possible postprandial effect of dietary calcium on these proteins in hamsters with a full stomach.

Effect of dietary calcium on sterol absorption is currently unknown. This study was the first time to demonstrate that dietary calcium deficiency could decrease the fecal excretion of not only acidic sterols but also neutral sterols (Table 3). Compared with that in the Ca-0 group, total neutral sterol excretion in Ca-2, Ca-6 and Ca-8 groups was increased by 68–80%, respectively. Most interestingly was that dietary calcium down-regulated the mRNA NPC1L1 and MTP, whereas it up-regulated the mRNA ABCG 5/8, suggesting a

decrease in cholesterol absorption associated with dietary calcium mediated by its effect on the gene expression of these transporters involved in neutral sterol absorption. We have also measured liver mRNA ABCG5/G8 and MTP, finding calcium at 6 and 8 g/kg diet up-regulated only ABCG5 but not ABCG8 and MTP. It is known that ABCG5/8 facilitates the secretion of liver sterols into bile and serve as an alternative mechanism, independent of intestinal ABCG5/G8, to protect against the accumulation of dietary sterols in plasma [29]. However, it was unclear how dietary calcium only up-regulated hepatic mRNA ABCG5 without any effect on hepatic mRNA ABCG8 and MTP.

This study found that plasma calcium concentrations increased slightly from 10.1 to 13.8 mg/dL when dietary calcium increased from 0.4 (Ca-0 group) to 8.4 g/kg diet (Ca-8 group), indicating that plasma calcium was not proportionally increased with that in diet. Result was in agreement with that of Dawson-Hughes *et al.* [30], who demonstrated plasma calcium in subjects given an additional of 500 mg calcium plus 700 IU vitamin D increased only by 2%. This is because plasma calcium concentration is tightly regulated by a complex homeostatic mechanism involving three major hormones: parathyroid hormone, calcitonin and 1,25-dihydroxyvitamin D [$1,25(OH)_2D_3$] [30, 31].

In summary, we found that dietary calcium favorably modified plasma lipoprotein profile by decreasing plasma TC, non-HDL-C, TC/HDL-C, non-HDL-C/HDL-C and TG levels, whereas increasing plasma HDL-C level in ovariectomized hamsters. Dietary calcium-induced reduction in plasma TC was accompanied by inhibition on intestinal ACAT activity, up-regulation of mRNA CYP7A1, down-regulation of mRNA NPC1L1 and MTP as well as up-regulation of intestinal mRNA ABCG 5/8. In addition, dietary calcium dose-dependently decreased plasma CETP activity, thus leading to a significant reduction in the ratio of non-HDL-C to HDL-C.

This project was supported by a grant from Hong Kong Research Grant Council (CUHK 4622/08M).

The authors have declared no conflict of interest.

5 References

[1] Hsu, H. H. T., Culley, N. C., Effects of dietary calcium on atherosclerosis, aortic calcification, and icterus in rabbits fed a supplemental cholesterol diet. *Lipids Health Dis.* 2006, *5*, 16.

[2] Pellizzon, M., Ana, J. S., Buison, E., Martin, J. *et al.*, Effect of a modified milk fat and calcium on cholesterol metabolism in hamsters. *Lipids* 2005, *39*, 441–448.

[3] Olatunji, L. A., Soladoye, A. O., Oyeyipo, P. I., Effect of increased dietary calcium on hemorheological, lipid and lipid peroxidation in oral contraceptive-treated female rats. *Clin. Hemorheol. Microcirc.* 2008, *38*, 135–142.

[4] Vaskonen, T., Mervaala, E., Sumuvuori, V., Seppänen-Laakso, T., Karppanen, H., Effects of calcium and plant sterols on serum lipids in obese Zucker rats on a low-fat diet. *Br. J. Nutr.* 2002, *87*, 239–245.

[5] De Rodas, B. Z., Gilliland, S. E., Maxwell, C. V., Hypocholesterolemic action of *Lactobacillus acidophilus* ATCC 43121 and calcium in swine with hypercholesterolemia induced by diet. *J. Dairy Sci.* 1996, *79*, 2121–2128.

[6] Jacqmain, M., Doucet, E., Despres, J. P., Bouchard, C., Tremblay, A., Calcium intake, body composition, lipoprotein-lipid concentration in adults. *Am. J. Clin. Nutr.* 2003, *77*, 1448–1452.

[7] Andryskowski, G., Chojnowska-Jezierska, J., Broncel, M., Barylski, M., Banach, M., Effect of calcium lactate supplementation on cholesterol concentration in patients with hyperlipidaemia and previous viral hepatitis: a preliminary report. *Cardiovasc. J. Afr.* 2008, *19*, 84–87.

[8] Reid, I. R., Mason, B., Horne, A., Ames, R. *et al.*, Effects of calcium supplementation on serum lipid concentration in normal older women: a randomized controlled trial. *Am. J. Med.* 2002, *112*, 343–347.

[9] Ditscheid, B., Keller, S., Jahreis, G., Cholesterol metabolism is affected by calcium phosphate supplementation in humans. *J. Nutr.* 2005, *135*, 1678–1682.

[10] Davis, H. R., Jr., Altmann, S. W., Niemann-Pick C1 Like 1 (NPC1L1) an intestinal sterol transporter. *Biochim. Biophys. Acta* 2009, *1791*, 679–683.

[11] Temel, R. E., Gebre, A. K., Parks, J. S., Rudel, L. L., Compared with acyl-CoA:cholesterol O-acyltransferase (ACAT) 1 and lecithin:cholesterol acyltransferase, ACAT2 displays the greatest capacity to differentiate cholesterol from sitosterol. *J. Biol. Chem.* 2003, *278*, 47594–47601.

[12] Brown, M. S., Goldstein, J. L., A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc. Natl. Acad. Sci. USA* 1999, *96*, 11041–11048.

[13] Zhang, Z. S., Wang, H., Jiao, R., Peng, C. *et al.*, Choosing hamsters but not rats as a model for studying plasma cholesterol-lowering activity of functional foods. *Mol. Nutr. Food Res.* 2009, *53*, 921–930.

[14] Lee, J. Y., Mitmesser, S. H., Car, T. P., in: Zempleni J., Daniel H. (Eds.), *Molecular Nutrition*, CABI Publishing, London 2002, pp. 309–320.

[15] Lam, C. K., Chen, J., Cao, Y., Yang, L. *et al.*, Conjugated and non-conjugated octadecaenoic acids affect differently intestinal acyl coenzyme A: cholesterol acyltransferase activity. *Atherosclerosis* 2008, *198*, 85–93.

[16] Chan, P. T., Fong, W. P., Cheung, Y. L., Huang, Y. *et al.*, Jasmine green epicatechins are hypolipidemic in hamsters fed a high fat diet. *J. Nutr.* 1999, *129*, 1094–1101.

[17] Yeung, T. C. H., Yang, L., Huang, Y., Wang, J., Chen, Z. Y., Dietary conjugated linoleic acid mixture affects the activity of intestinal acyl coenzyme A: cholesterol acyltransferase in hamsters. *Br. J. Nutr.* 2000, *84*, 935–941.

[18] Vaziri, N. D., Liang, K. H., Down-regulation of hepatic LDL receptor expression in experimental nephrosis. *Kidney Int.* 1996, *50*, 887–893.

[19] Valasek, M. A., Repa, J. J., Quan, G., Dietschy, J. M., Turley, S. D., Inhibiting intestinal NPC1L1 activity prevents diet-induced increase in biliary cholesterol in Golden Syrian hamsters. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2008, *295*, 813–822.

[20] Field, F. J., Born, E., Mathur, S. N., Stanol esters decrease plasma cholesterol independently of intestinal ABC sterol transporters and Niemann-Pick C1-like 1 protein gene expression. *J. Lipid Res.* 2004, *45*, 2252–2259.

[21] Qin, B., Qiu, W., Avramoglu, R. K., Adeli, K., Tumor necrosis factor-alpha induces intestinal insulin resistance and stimulates the overproduction of intestinal apolipoprotein B48-containing lipoproteins. *Diabetes* 2007, *56*, 450–461.

[22] Lien, E. I., Boyle, F. G., Wrenn, J. M., Perry, R. W. *et al.*, Comparison of AIN-76A and AIN-93G diet. *Food Chem. Toxicol.* 2001, *39*, 385–392.

[23] Reid, I. R., Effects of calcium supplementation on circulating lipids: potential pharmacoeconomic implications. *Drugs Aging* 2004, *21*, 7–17.

[24] Barter, P. J., Kastelein, J. J., Targeting cholesteryl ester transfer protein for the prevention and management of cardiovascular disease. *J. Am. Coll. Cardiol.* 2006, *47*, 492–499.

[25] Doggrell, S. A., Inhibitors of cholesteryl ester transfer protein – a new approach to coronary artery disease. *Expert Opin. Invest. Drugs* 2006, *15*, 99–106.

[26] Dullaart, R. P., Dallinga-Thie, G. M., Wolffenbuttel, B. H., van Tol, A., CETP inhibition in cardiovascular risk management: A critical appraisal. *Eur. J. Clin. Invest.* 2007, *37*, 90–98.

[27] Okamoto, H., Miyai, A., Sasase, T., Furukawa, N. *et al.*, Cholesteryl ester transfer protein promotes the formation of cholesterol-rich remnant like lipoprotein particles in human plasma. *Intl. J. Clin. Chem.* 2007, *375*, 92–98.

[28] Greenough, M. C., Boegman, R. J., 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase in normal and dystrophic hamsters. *Biochem. J.* 1982, *201*, 501–504.

[29] Wu, J. E., Basso, F., Shamburek, R. D., Amar, M. J. A. *et al.*, Title: Hepatic ABCG5 and ABCG8 overexpression increases hepatobiliary sterol transport but does not alter aortic atherosclerosis in transgenic mice. *J. Biol. Chem.* 2004, *279*, 22913–22925.

[30] Dawson-Hughes, B., Harris, S. S., Krall, E. A., Dallal, G. E., Effect of calcium and vitamin D supplementation on bone density in men and women 65 years of age or older. *N. Engl. J. Med.* 1997, *337*, 670–676.

[31] Mundy, G. R., Guise, T. A., Hormonal control of calcium homeostasis. *Clin. Chem.* 1999, *45*, 1347–1452.

