Effect of Proanthocyanidins and Consumption Frequency of Sterols and Fatty Acids on Lipoprotein Metabolism in Hamsters

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Abstract

Hypercholesterolemia is one of the major proven risk factors for atherosclerosis. Decreasing blood total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels with cholesterol-lowering nutraceuticals and dietary intake modification could slow or reverse the progression of cardiovascular disease.

Grape seed proanthocyanidins (GSP) as a cholesterol-lowering nutraceutical has been investigated in both humans and animals, however, little is known of how it interacts with the genes and proteins involved in lipoprotein metabolism *in vivo*. So the first objective of the present study was to investigate the effect of GSP supplementation on blood cholesterol level and gene expression of cholesterol-regulating enzymes in Golden Syrian hamsters maintained on a 0.1% cholesterol diet.

The second objective of the present study was to investigate the effect of cholesterol, myristic acid and β-sitosterol consumption frequency on plasma lipoprotein profiles in hamsters. Numerous studies reported that dietary cholesterol and saturated fatty acids elevated plasma TC level, whereas dietary phytosterols in moderate and high doses favorably reduced plasma TC and LDL levels. However, it is still unknown whether consumption frequency of sterols and fatty acids could affect plasma cholesterol level and lipid profiles.

The results affirmed supplementation of 0.5% or 1.0% GSP could decrease

plasma TC, non-high density lipoprotein cholesterol (Non-HDL-C) and triglyceride (TG) levels. In addition, dietary GSP was able to increase the excretion of bile acids by 3-4 folds, this was partially mediated by up-regulation of Cholesterol 7 alpha-hydroxylase (CYP7A1) in both transcriptional and translational levels. It was concluded that the hypocholesterolemic activity of GSP was most likely mediated by enhancement of bile acid excretion and up-regulation of CYP7A1.

In the cholesterol consumption frequency study, hamsters were given daily 9 mg of cholesterol either in diet (high cholesterol intake frequency) or a gavage-administration of 3 times 3 mg (regular cholesterol intake frequency) and 1 time 9 mg (low cholesterol intake frequency). The results demonstrated that there was an increasing trend in concentrations of plasma TC, Non-HDL-C, TC/HDL-C ratio and TG in association with the cholesterol intake frequency. It is the first time to demonstrate that the increasing cholesterol intake frequency increased the apparent cholesterol absorption. Elevation of plasma TC and cholesterol absorption is most likely mediated by up-regulation of intestinal Niemann-Pick C1-like 1 (NPC1L1), acyl coenzyme A: cholesterol acyltransferase 2 (ACAT2), and microsomal triacylglycerol transport protein (MTP) gene expression.

In the myristic acid consumption frequency study, hamsters were given daily 210 mg of myristic acid either in diet (high myristic acid intake frequency) or a gavage-administration of 3 times 70 mg (regular myristic acid intake frequency) and 1 time 210 mg (low myristic acid intake frequency). The results showed that the increasing consumption frequency elevated plasma TC, Non-HDL-C and HDL-C

levels. Elevation of plasma TC and HDL-C is most likely mediated by up-regulation of NPC1L1 and down-regulation of scavenger receptor BI (SR-BI) gene expression via enhancement of dietary myristic acid absorption.

In the β-sitosterol consumption frequency study, hamsters fed the basal diet with a gavage-administration of 3 mg cholesterol 3 times (control), or a gavage-administration of 3 mg β-sitosterol with 3 mg cholesterol 3 times per day (high β-sitosterol intake frequency), or a gavage-administration of 9 mg β-sitosterol with 3 mg cholesterol for one time and 3mg cholesterol for the other two times (low β-sitosterol intake frequency). The results demonstrated that for a given dose of β-sitosterol, the administration frequency had no or little effect on plasma lipoprotein profiles. The present study also found that cholesterol-lowering activity of β-sitosterol was mediated by its inhibition on the intestinal cholesterol absorption with up-regulation of NPC1L1, ATP binding cassette transporters G5 and G8 (ABCG5/8) and MTP.

In conclusion, the present study confirmed that hypocholesterolemic activity of GSP was most likely mediated by enhancement of bile acid excretion and up-regulation of CYP7A1. The present study also demonstrated that frequent cholesterol and myristic acid intake is associated with elevation of plasma TC level, while β-sitosterol intake frequency had no effect on plasma cholesterol for a given amount.

// 摘要

高膽固醇血症是導致動脈粥樣硬化的主要原因之一。攝食降膽固醇的營養品以及合理調整膳食方式可有效降低血液總膽固醇和低密度脂蛋白，從而達到防治心血管疾病的效果。

葡萄籽原花青素作爲降膽固醇的營養品已經在人體及動物體內得到廣泛研究。然而體內試驗中，其如何調控脂蛋白代謝相關的基因和蛋白卻尚未清楚。所以，本實驗的第一個目的就是研究葡萄籽原花青素對餵食 0.1%高膽固醇糧的倉鼠血膽固醇及膽固醇調解相關基因表達的影響。

本試驗第二個目的是研究膽固醇、肉豆蔻酸及 β-穀甾醇的攝食頻率對倉鼠血脂蛋白的影響。大量的實驗表明攝食膽固醇及飽和脂肪酸可以增加血漿總膽固醇量，然而中等劑量及高劑量的植物甾醇可以有效降低血漿總膽固醇和低密度脂蛋白量。但目前爲止，固醇及脂肪酸的攝食頻率對血膽固醇及血脂的影響卻尚未確定。

實驗結果表明在飼料裏添加 0.5%或 1% 的葡萄籽原花青素可以有效降低血漿總膽固醇、非高密度脂蛋白膽固醇及甘油三酯量。並且，葡萄籽原花青素可以增加 3-4 倍的膽汁酸排泄，這有可能是上調膽固醇 7α-羥化酶（CYP7A1）的基因及蛋白量造成的。從而得到的結論是葡萄籽原花青素的降膽固醇活性主要與增加膽汁酸排出及上調 CYP7A1 有關。

在膽固醇攝食頻率的研究中，所有倉鼠每天都攝入 9 毫克膽固醇：將膽固醇添加在糧裏（高頻率攝食膽固醇）；或者分三次每次灌喂 3 毫克膽固醇（中頻率攝食膽固醇）；或者一次性灌喂 9 毫克膽固醇（低頻率攝食膽固醇）。研究結果發現，隨著膽固醇攝入頻率增高，血漿總膽固醇、非高密度脂蛋白膽固醇、總膽固醇/高密度脂蛋白膽固醇比值及甘油三酯也隨之上升。並且本研究首次發

現增加膽固醇攝食頻率可以促進膽固醇的吸收。而增加血漿總膽固醇及膽固醇吸收則是通過上調腸內 Niemann-Pick C1-like 1（NPC1L1）、醯基輔酶 A：膽固醇醯基轉移酶 2（ACAT2）及微粒三酸甘油酯轉移蛋白（MTP）的基因表達來完成的。

在肉豆蔻酸攝食頻率研究中，所有倉鼠每天都攝入 210 毫克肉豆蔻酸：將肉豆蔻酸添加在糧裏（高頻率攝食肉豆蔻酸）；或者分三次每次灌喂 70 毫克肉豆蔻酸（中頻率攝食肉豆蔻酸）；或者一次性灌喂 210 毫克肉豆蔻酸（低頻率攝食肉豆蔻酸）。研究結果表明，若膳食中攝入等量的肉豆蔻酸，高頻率的攝入方式可以增加血漿總膽固醇、非高密度脂蛋白膽固醇及高密度脂蛋白膽固醇量。升高血漿總膽固醇和高密度脂蛋白膽固醇主要是通過上調 NPC1L1 及下調清道夫受體 B 類 I 型（SR-BI）的基因表達從而加強膳食中對肉豆蔻酸的吸收來完成的。

在β-穀甾醇攝食頻率研究中，倉鼠每天灌喂3次3毫克膽固醇（對照組）；或者每天灌喂三次3毫克β-穀甾醇加3毫克膽固醇（高頻率攝食β-穀甾醇）；或者每天一次性灌喂9毫克β-穀甾醇加3毫克膽固醇，另兩次分別只灌喂3毫克膽固醇（低頻率攝食β-穀甾醇）。研究結果發現等量β-穀甾醇不同頻率的攝入方式對血漿脂蛋白的變化情況並無大的影響。不僅如此，本研究還發現β-穀甾醇降膽固醇的活性是由於其可以通過上調 NPC1L1、ATP binding cassette transporters G5 and G8 (ABCG5/8) 及 MTP從而達到抑制腸膽固醇吸收的目的。

綜上所述，本研究證實了葡萄籽原花青素的降膽固醇活性主要與增加膽汁酸排出及上調 CYP7A1 有關。並且，還發現提高膽固醇及肉豆蔻酸的攝食頻率可增加血漿總膽固醇量，然而等量 β-穀甾醇不同頻率的攝入方式並不影響血膽固醇。

List of Abbreviations

ABCG5	ATP binding cassette transporters sub-family G member 5
ABCG8	ATP binding cassette transporters sub-family G member 8
ACAT2	acyl-CoA: cholesterol acyltransferase 2
apoA-I	apolipoprotein A-I
ATP	adenosine-triphosphate
cDNA	complementary DNA
CE	cholesteryl ester
CEH	cholesteryl ester hydrolase
CHD	coronary heart disease
CM	chylomicron
CYP7A1	cholesterol-7α-hydroxylase
FATP	fatty acid transport protein
FXR	farnesoid X receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSP	Grape seed proanthocyanidins
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
HMG-CoA-R	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPLC	high performance liquid chromatography
IDL	intermediate-density lipoprotein
LCAT	lecithin-cholesterol acyltransferase
LDL	low-density lipoprotein

LDL-C	low-density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor
LXR	liver X receptor
MTP	microsomal triglyceride transfer protein
NADPH	nicotinamide adenine dinucleotide phosphate
NPC1L1	Niemann-Pick C1 like 1
PPAR	peroxisome proliferator activated receptor
PVDF	polyvinylidene difluoride
RCT	reverse cholesterol transport
RXR	retinoid X receptor
SFA	saturated fatty acid
SR-BI	scavenger receptor B type I
SREBP-2	sterol regulatory element-binding protein 2
TC	total cholesterol
TG	triglyceride
VLDL	very low-density lipoprotein

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Chapter 1

General Introduction

1.1 Cholesterol and its chemical structure

Cholesterol is a waxy sterol found in the cell membranes and transported in the blood plasma of all animals. Cholesterol is the principal sterol synthesized by animals, and also very small quantities are synthesized in other eukaryotes, such as plants and fungi (Janson et al., 2009). However, it is almost completely absent among prokaryotes, which include bacteria (Pearson et al., 2003).

Since cholesterol is insoluble in water, it is carried in the blood among tissues by four lipoproteins: chylomicron (CM), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). CM, formed in the intestinal lymphatics, can transport dietary cholesterol and triglycerides (TG) from the intestine to adipose tissue and skeletal muscles (Brown et al., 1983). VLDL is produced in the liver and carries the newly synthesized TG and cholesterol from the liver to adipose tissue and skeletal muscles. LDL, a major cholesterol carrier in blood, is formed in plasma when intermediate-density lipoprotein (IDL) acquires cholesteryl ester (CE) from HDL (Walker et al., 1994). LDL provides cholesterol to those tissues that need it. HDL removes excessive cholesterol from peripheral tissues back to the liver where cholesterol is converted to bile acids for excretion. In this regard, HDL plays a major role in maintaining cholesterol homeostasis in the blood. In general, LDL is considered "bad" cholesterol, whereas HDL is often regarded as "good" cholesterol (Diepeveen et al., 2008).

Cholesterol is an isoprenoid molecule which possesses 27 carbon atoms and is composed of three regions: a ring structure region with 4 hydrocarbon rings, a

hydrocarbon side chain at C17 and a hydroxyl group at C3 (see Figure 1.1). Cholesterol is a very hydrophobic molecule, with only limited polarity due to the hydroxyl (OH) group. Both the ring region and tail region on cholesterol are non-polar, meaning cholesterol dissolves in fatty and oily substances but it will not mix with water. The hydrophobicity of cholesterol is, on the one hand, responsible for its beneficial property to control cell membrane fluidity; but on the other hand, it makes cholesterol very difficult to transport in the aqueous environment of the body, both within cells and between cells.

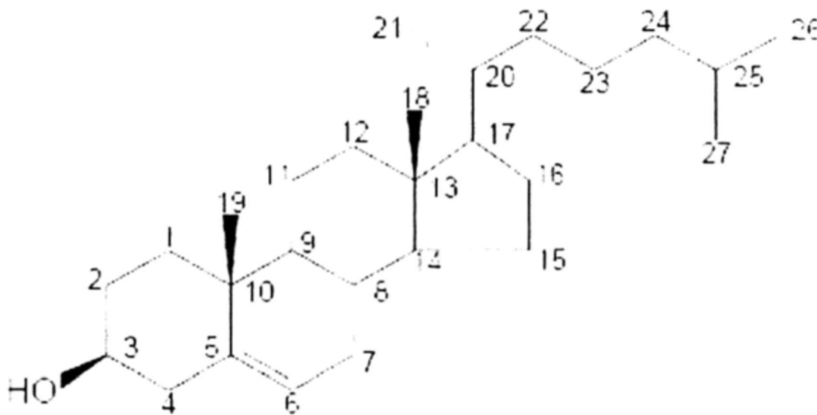


Figure 1.1

Chemical structure of cholesterol with the standard carbon numbering according to IUPAC recommendations.

1.2 Functions of cholesterol in mammals

Cholesterol has a number of functions. It is a structural component of cell membranes and regulates membrane fluidity over the range of physiological temperatures. The bulky steroid and the hydrocarbon chain of cholesterol are embedded in the membrane alongside the nonpolar fatty acid chain of the other lipids, while the hydroxyl group on cholesterol interacts with the polar head groups of the membrane phospholipids and sphingolipids (Kurzchalia et al., 2003). In this

structural role, cholesterol reduces the permeability of the plasma membrane to sodium ions and protons (positive hydrogen ions) (Haines, 2001).

Within the cell membrane, cholesterol also functions in intracellular transport, cell signaling and nerve conduction. Cholesterol is the precursor molecule in several biochemical pathways. In the liver, cholesterol is converted to bile acids, which are then stored in the gallbladder. Bile contains bile salts, which solubilize fats in the digestive tract and aid in the intestinal absorption of fat molecules as well as the fat-soluble vitamins, Vitamin A, Vitamin D, Vitamin E, and Vitamin K. Besides, cholesterol is an important precursor molecule for the synthesis of Vitamin D and the steroid hormones, including the adrenal gland hormones, cortisol and aldosterone as well as the sex hormones progesterone, estrogens, and testosterone, and their derivatives (Daniels et al., 2009).

1.3 Harmful effects of cholesterol: Atherosclerosis

The plasma cholesterol level in particularly LDL-associated cholesterol level is one of the main risk factors of atherosclerosis, and other risk factors includes cigarette smoking, hypertension, obesity, diabetes, sedentary lifestyle, age, male sex, and heredity (Chang et al., 2010). In the United States and most other developed countries, atherosclerosis is the leading cause of illness and death. Estimates for 2005 in the United States alone are that about 16 million people have atherosclerotic heart disease and 5.8 million people have stroke (Lam, 2008). Cardiovascular disease, primarily coronary and cerebrovascular atherosclerosis, caused almost 870,000 deaths, almost twice as many as cancer caused and 9 times as many as injuries caused in 2005 (Lam, 2008).

Briefly, atherosclerotic lesions develop as follows. Firstly, any chemical or mechanical injury can damage the endothelial cell layer (NexVas™, 2010). This

alters the normal blood flow and provides sites for the adhesion and aggregation of platelet, leading to the formation of blood clots or thrombi in the arterial wall. Alterations of the endothelial layer cause white blood cells (macrophages) to stick and to migrate into this layer, where they become active macrophages. Once macrophages make their way into the inner endothelial layer they begin to convert and accumulate modified LDL cholesterol. This modified LDL cholesterol results in a foam cell formation in macrophages. Secondly, the earliest recognizable lesions of the inner arterial layer are called "fatty streaks", which are aggregations of foam cells. Foam cells induce the further replacement of smooth endothelial cells by muscle cells from the medium arterial wall layer. These cells convert into cells that produce connective tissue and even take up lipids themselves. Thirdly, in these damaged sites, there begin a cycle of inflammation and proliferation, leading to further platelet adhesion and aggregation, leading to the formation of thrombi in the arterial wall. Over time accumulation of fatty streaks stimulates continued cell influx, cholesterol deposition, smooth muscle cell expansion and connective tissue formation, all of which lead to more advanced lesions. These lesions, called fibrous plaques, cling to the inner arterial side and increase in size, thus narrowing the arterial lumen. Fibrous plaques are soon covered by a thick dome of connective tissue with embedded smooth muscle cells that usually overlay a core of lipid and necrotic debris. Lastly, with time the plaques become calcified and may undergo further changes leading to a partial reduction or total block of the blood's flow through an artery. If blood is unable to flow through the vessels, it cannot nourish the tissues. Eventually a blood clot forms and stops blood flow to the heart muscle. The result is heart attack and death of portion of heart muscle (Figure 1.2)

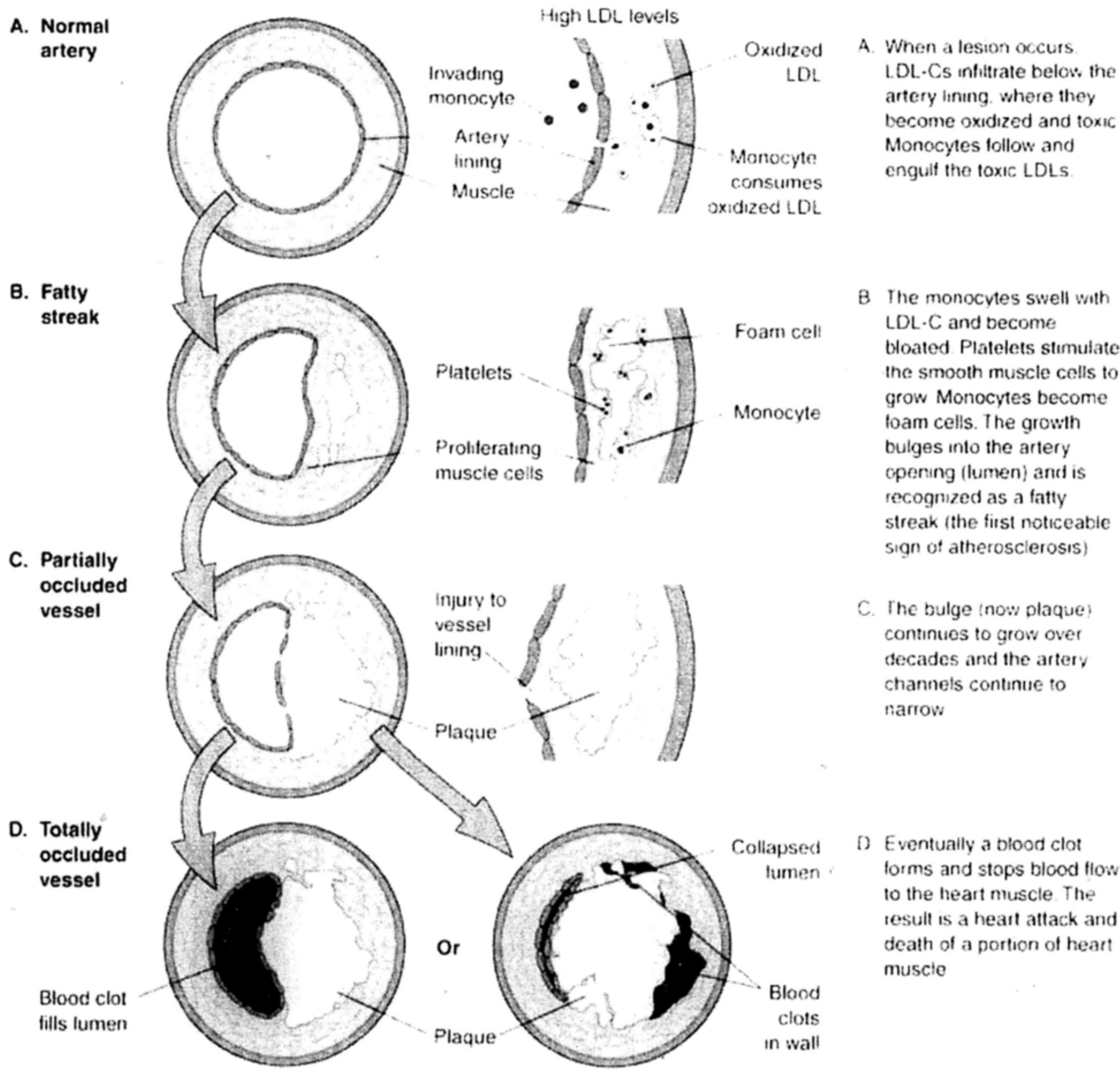


Figure 1.2

The development of atherosclerosis (http://s293.photobucket.com/albums/mm53/nexavar/atherosclerosis/?action=view¤t=Atherosclerosis_process_jpg.jpg)

1.4 Cholesterol homeostasis

Epidemiological studies have demonstrated that elevated levels of plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) are the major risk factors, while high concentrations of plasma high-density lipoprotein cholesterol (HDL-C) and a low ratio of TC to HDL-C are protective ones against coronary heart disease (Sharrett et al., 2001). For many years, liver and small intestine are considered the most important organs for maintenance of whole body cholesterol homeostasis. While liver is the main site for cholesterol de novo synthesis, degradation and excretion in bile fluid as bile acids, it receives most of the cholesterol absorbed by the small intestine, clears cholesterol-containing CM remnants and low density lipoprotein particles from plasma and is the major contributor to high density lipoprotein formation (Kruit et al., 2006). Cholesterol homeostasis is maintained at the transcriptional level by sterol regulatory element-binding protein 2 (SREBP-2) and liver X receptor alpha (LXRα) in a coordinated manner (Lee et al., 2002). SREBP-2 governs the transcription of 3-hydroxy-3-methylglutaryl reductase (HMG-CoA-R) and low-density lipoprotein receptor (LDLR), with HMG-CoA-R acting as the rate-limiting enzyme in cholesterol synthesis whereas LDL-R being responsible for the removal of LDL-C from the circulation. LXRα regulates the transcription of a gene encoding cholesterol-7α-hydroxylase (CYP7A1), which converts cholesterol to bile acids for elimination (Lee et al., 2002). During the past couple of years, small intestine in maintenance of cholesterol homeostasis and regulation of plasma cholesterol levels has been paid more and more attention. Small intestine is the key target for cholesterol absorption, cholesterol excretion and plasma HDL cholesterol levels (Kruit et al., 2006). In a typical western diet consumed by the average adult, about 1,200-1,700 mg of cholesterol enters the lumen of the small intestine each day

(Grundy, 1983). Nearly 300-500 mg of this cholesterol comes from the diet and the remainder is mainly derived from bile (Grundy et al., 1972). The mechanism for cholesterol absorption as well as intestinal sterol excretion is partially known. First, cholesterol and phytosterols are absorbed into the enterocytes by Niemann-Pick C1 like 1 (NPC1L1) (Davis et al., 2004). Then phytosterols effectively compete with cholesterol pumped back into intestinal lumen by ATP binding cassette transporters sub-family G member 5 and 8 (ABCG5/8) for fecal excretion (Lee et al., 2001 & Berge et al., 2000). Second, acyl-CoA: cholesterol acyltransferase 2 (ACAT2) is highly expressed in enterocytes in human and is the predominant enzyme responsible for the intracellular esterification of cholesterol and their subsequent secretion with lipoproteins. Then microsomal triglyceride transfer protein (MTP) transfers cholesterol esters from the ER membranes to nascent ApoB-lipoprotein for assembly of chylomicrons in enterocytes (Hussain et al., 2000 & Atzel et al., 1993) (Figure 1.3).

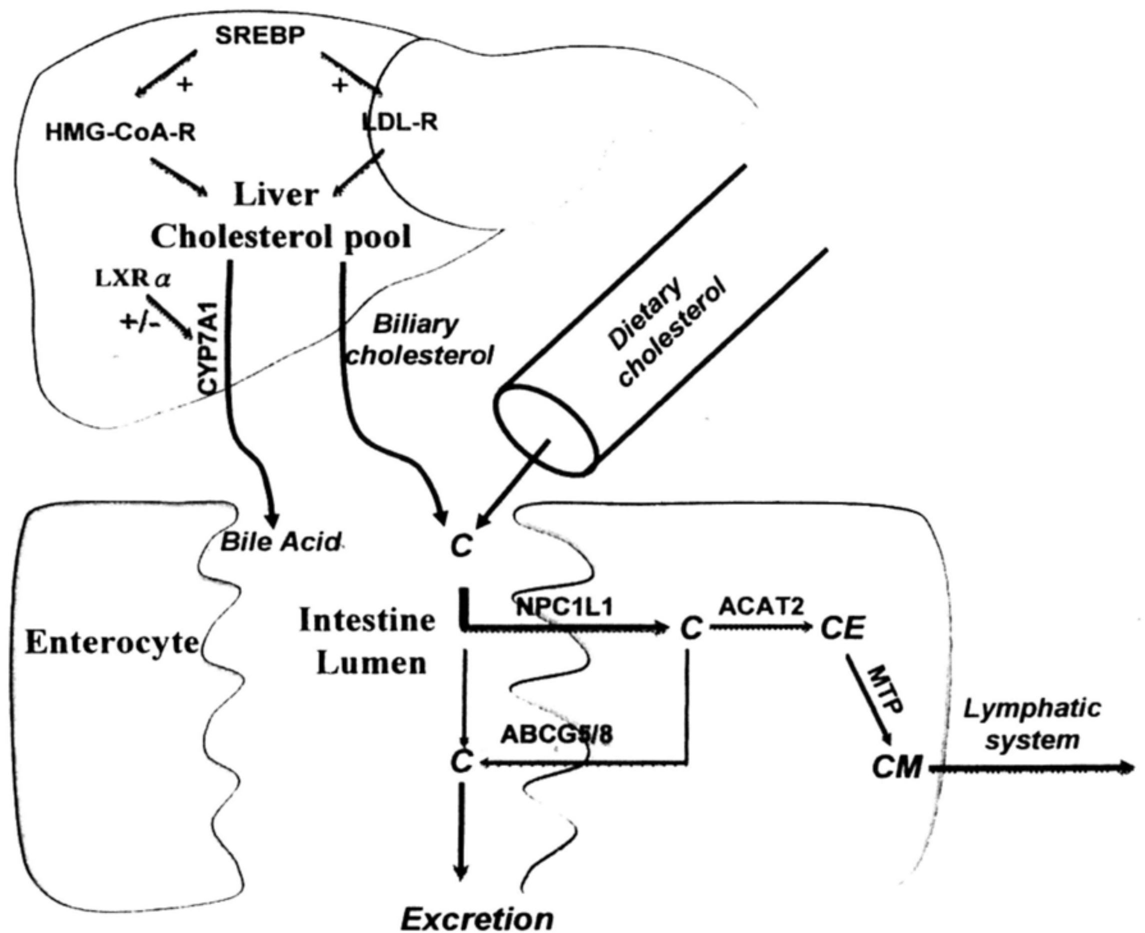


Figure 1.3

Cholesterol homeostasis. Cholesterol in the lumen is derived from two sources namely diet and bile. SREBP-2 regulates the gene expression of LDLR and HMG-CoA-R, whereas LXRα governs the gene expression of CYP7A1. Cholesterol is transported into enterocytes via intestinal NPC1L1. Intestinal ACAT2 esterifies cholesterol to form CE, which is packed with MTP into CM and transferred into blood through the lymphatic system. ABCG5/8 return minor amount of unabsorbed cholesterol to the lumen of the intestine for excretion. +, U-regulation. -, Down-regulation (Jiao et al., 2010).

1.4.1 SREBPs

In vertebrate cells, lipid homeostasis is regulated by a family of membrane-bound transcription factors designated SREBPs. SREBPs directly activate the expression of more than 30 genes devoted to the synthesis and uptake of cholesterol, triglycerides, fatty acids and phospholipids, as well as the nicotinamide adenine dinucleotide phosphate (NADPH) cofactor required to synthesize these molecules (Brown et al., 1997 & Horton et al., 1999 & Edwards et al., 2000 & Sakakura, 2001). The mammalian genome encodes three SREBP isoforms: SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1a is a mighty activator of all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, triglycerides, and fatty acids (Horton et al., 2002). SREBP-1c preferentially improves transcription of genes required for fatty acid synthesis (Horton et al., 2002). Although SREBP-2 has a long transcriptional activation domain, it preferentially activates cholesterol synthesis but not fatty acid synthesis (Brown et al., 1997). SREBP-1c and SREBP-2 predominant in the liver and most other intact tissues, whereas SREBP-1a and SREBP-2 are the predominate isoforms of SREBP in most cultured cell lines (Shimomura et al., 1997).

SREBP-2 activates cholesterologenesis while SREBP-1c activates the fatty acid biosynthetic pathway (Figure 1.4). SREBP-2–responsive genes in the cholesterol biosynthetic pathway include HMG-CoA synthase, HMG-CoA-R, farnesyl diphosphate synthase, and squálene synthase (Horton et al., 2002). SREBP-1c–responsive genes include those for ATP citrate lyase which produces fatty acid synthase which together produce palmitate (C16:0) and acetyl-CoA and acetyl-CoA carboxylase (Horton et al., 2002). Other SREBP-1c target genes encode a rate-limiting enzyme of the fatty acid elongase complex, which converts palmitate (C16:0) to stearate (C18:0) (Moon et al., 2001); stearoyl-CoA desaturase, which

converts stearate to oleate (C18:1); and glycerol-3-phosphate acyltransferase, the first committed enzyme in triglyceride and phospholipid synthesis (Edwards et al., 2000). Eventually, SREBP-1c and SREBP-2 activate three genes required to generate NADPH, which is used at multiple stages in these lipid biosynthetic pathways (Shimomura et al., 1998).

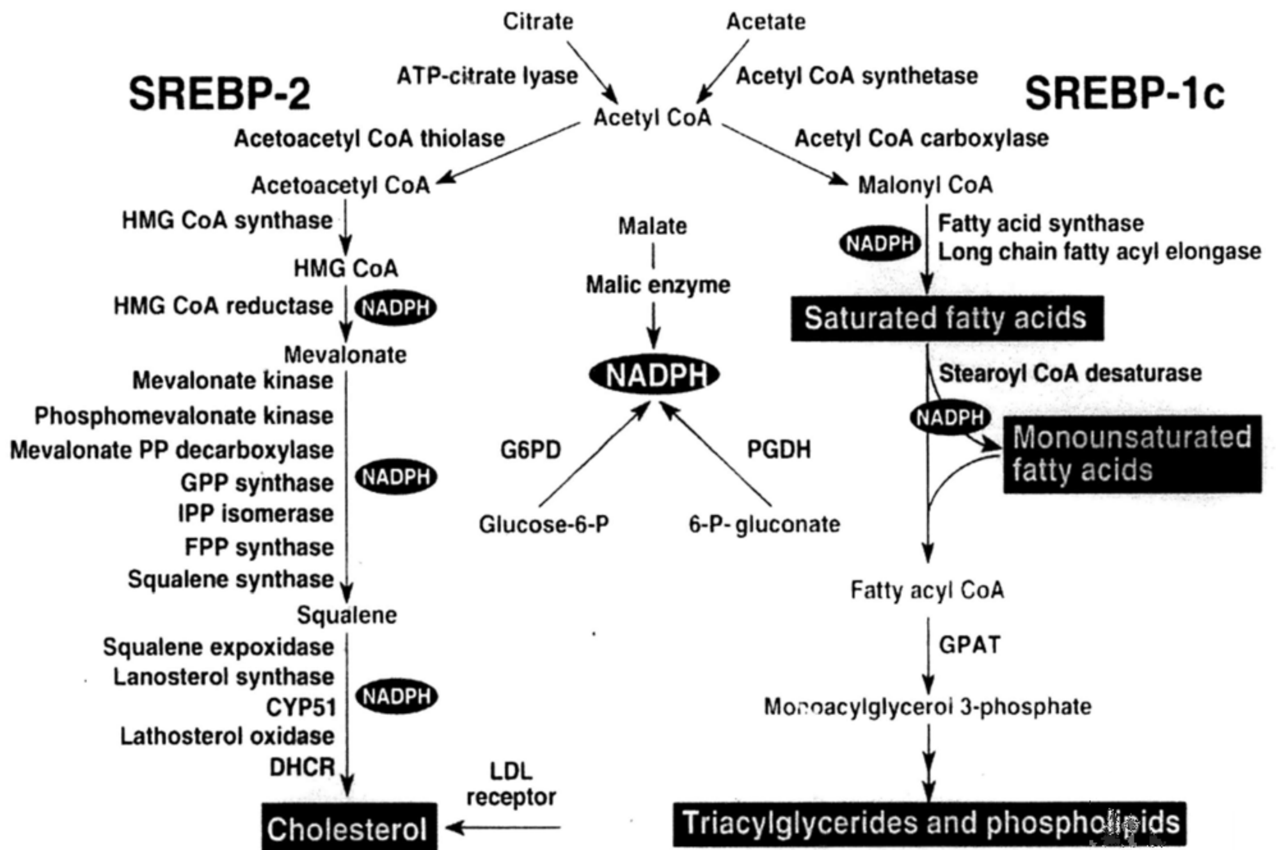


Figure 1.4

Genes regulated by SREBPs. The diagram shows the major metabolic intermediates in the pathways for synthesis of cholesterol, triglycerides and fatty acids. GPP, geranylgeranyl pyrophosphate synthase; FPP, farnesyl diphosphate; CYP51, lanosterol 14α-demethylase; DHCR, 7-dehydrocholesterol reductase; G6PD, glucose-6-phosphate dehydrogenase; PGDH, 6-phosphogluconate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase (Horton et al., 2002).

1.4.2 HMG-CoA-R

HMG-CoA-R is the rate-limiting enzyme of the mevalonate pathway, the metabolic process that produces cholesterol and other isoprenoids which are vital for diverse cellular functions, ranging from cholesterol synthesis to growth control (Figure 1.7) (Goldstein et al., 1990). HMG-CoA-R, anchored in the membrane of the endoplasmic reticulum, has eight transmembrane domains, with the active site being located in a long carboxyl terminal domain in the cytosol (Roitelman et al., 1992). Inhibition of cholesterol synthesis is the most efficient way to reduce serum cholesterol level. The discovery of the statin class of drugs (simvastatin and pravastatin) was a significant advance in the treatment of severe hypercholesterolemia. These drugs inhibit HMG-CoA-R in the liver. However, side effects are associated with the use of statin, including rashes and gastrointestinal symptoms (Walker, 1994).

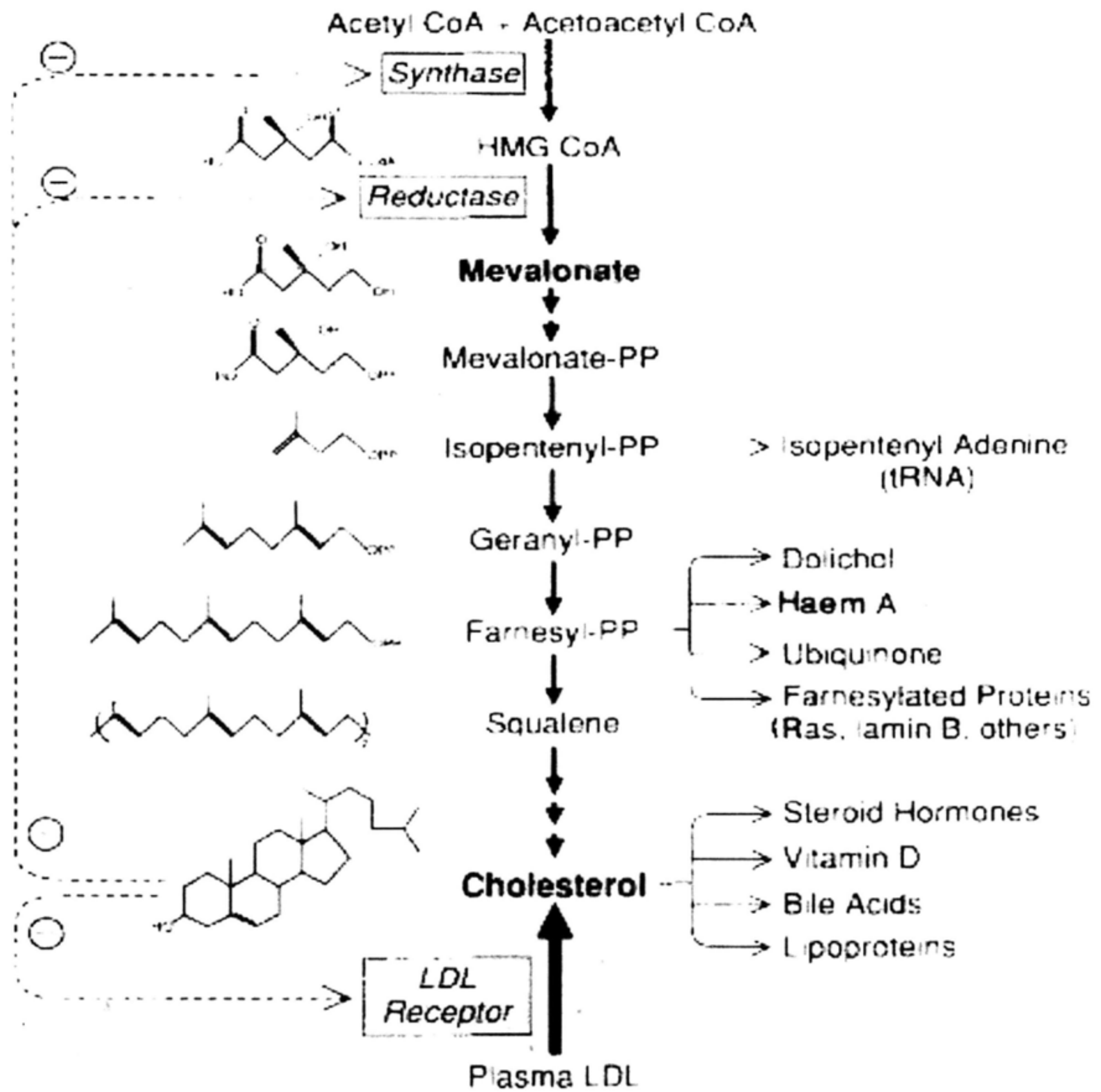


Figure 1.7

The mevalonate pathway in animal cells. The bulk product of mevalonate pathway, cholesterol, is obtained from two sources: (1) endogenously, by synthesis from acetyl-CoA through mevalonate; and (2) exogenously, from LDL receptor-mediated uptake of plasma LDL (Goldstein et al., 1990).

1.4.3 LDLR

LDLR is a mosaic protein that mediates the endocytosis of LDL. The principal ligand for the receptor is ApoB-100 on LDL, however, LDLR can catalyze endocytosis of lipoproteins containing multiple copies of ApoE such as VLDL, IDL, and HDL (Daniels et al., 2009).

Figure 1.8 describes the intracellular process in LDL endocytosis. LDLR positioning and subsequent endocytosis of the receptor-ligand complex happens at clathrin coated pits (Anderson et al., 1978). After endocytosis, acidic conditions of the endosome catalyze disassociation of LDL from the LDLR. Genetic studies have implicated that the epidermal growth factor precursor protein-like domain (EGFP) of LDLR as responsible for LDL release, as deletion of this region produces a non-separable complex (Davis et al., 1987). After release of the lipoprotein, the LDLR peptide is recycled back to the membrane in a process also commanded by the EGFP domain (Davis et al., 1987). Meanwhile, LDL particles that are released from the receptor fuse into lysosomes, and are degraded into lipid components and amino acids by enzymes of the vesicle. Large portions of lipids released are cholesteryl esters, which are hydrolyzed into free cholesterol by lysosomal acid lipase (LIPA) (Daniels et al., 2009). Cholesterol that has been endocytosed and converted to free form is often incorporated into cell membranes. However, depending on cell type, the free cholesterol has several other possible fates including efflux to cellular adaptors, metabolism into bile acids, synthesis of steroids or conversion back into cholesterol esters (Liscum et al., 1995). Expression of LDLR is a function of cellular free cholesterol. When the cellular cholesterol decreases, the LDL receptor gene is transactivated. In contrast, ample cellular free cholesterol down-regulates the LDL receptor will lead to a lower blood cholesterol level (Chen et al., 2008).

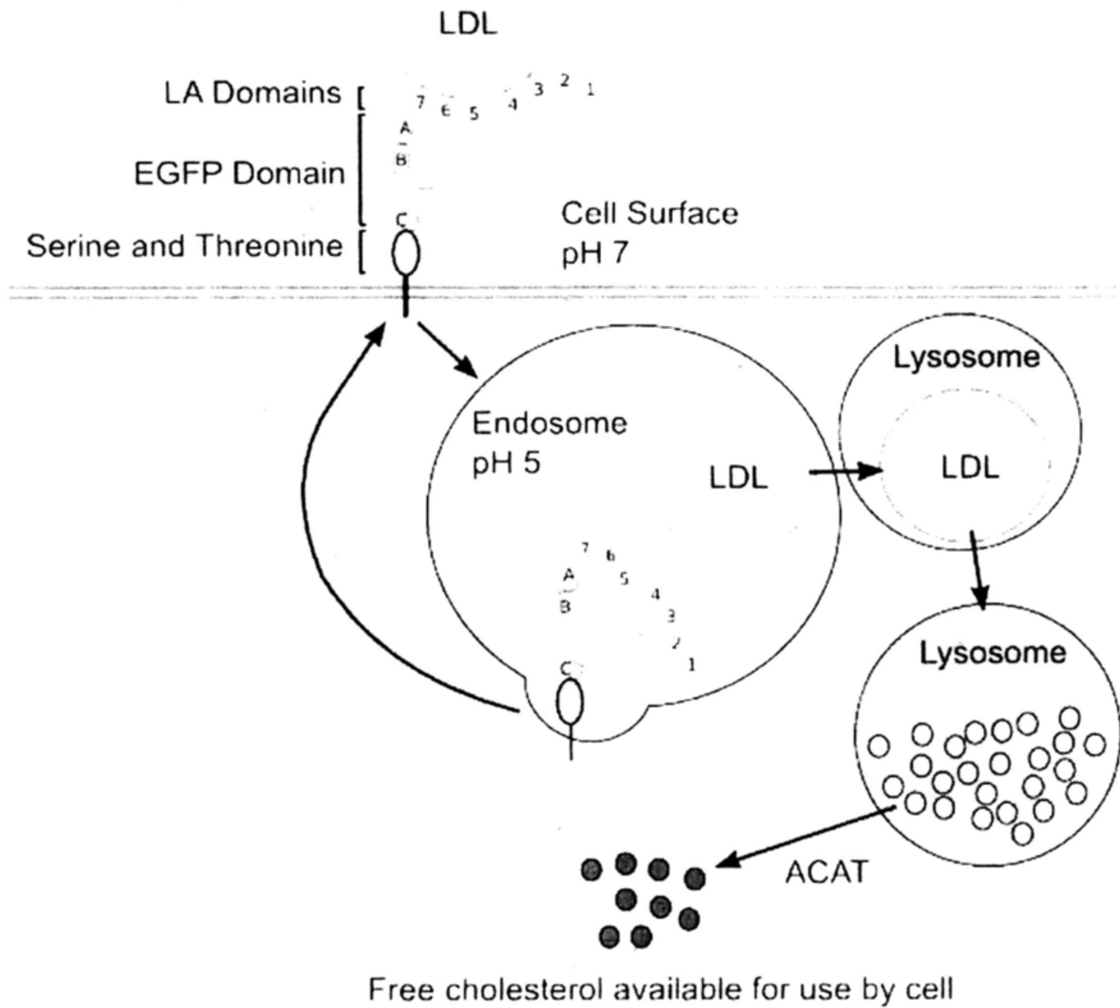


Figure 1.8

The LDL-R structure contains three types of domain: LDLR-type A domains (LA), the epidermal growth factor precursor protein-like domain (EGPF), and a domain rich in serine and threonine residues (Daniels et al., 2009).

1.4.4 LXRs

LXRs are ligand-activated transcription factors that belong to the nuclear receptor super-family. The LXR subfamily consists of two isoforms, LXRα (NR1H3) and LXRβ (NR1H2). These two isoforms are extremely related and share ~78% identity of their amino acid sequences in both DNA and ligand-binding domains (Ulven et al., 2005). High expression of LXRα is localized in the liver, spleen, adipose tissue, intestine, kidney and lung whereas LXRβ is expressed in most tissues (Apfel et al., 1994 & Song et al., 1994 & Willy et al., 1995 & Repa et al., 2000). The nuclear receptor heterodimers of LXR and retinoid X receptor (RXR) are key transcriptional regulators of genes involved in lipid homeostasis and inflammation (Svensson et al., 2003). Figure 1.5 shows that LXR response elements (LXREs) consists of two idealized hexanucleotide sequences (AGGTCA) separated by four bases (DR-4 element). So LXR/RXR is called a "permissive heterodimer" that may be activated by ligands for either partner in an independent manner (Willy et al., 1995). In the absence of ligands LXR recruits complexes of corepressors that are exchanged with coactivators upon receptor activation (Wojcicka et al., 2007).

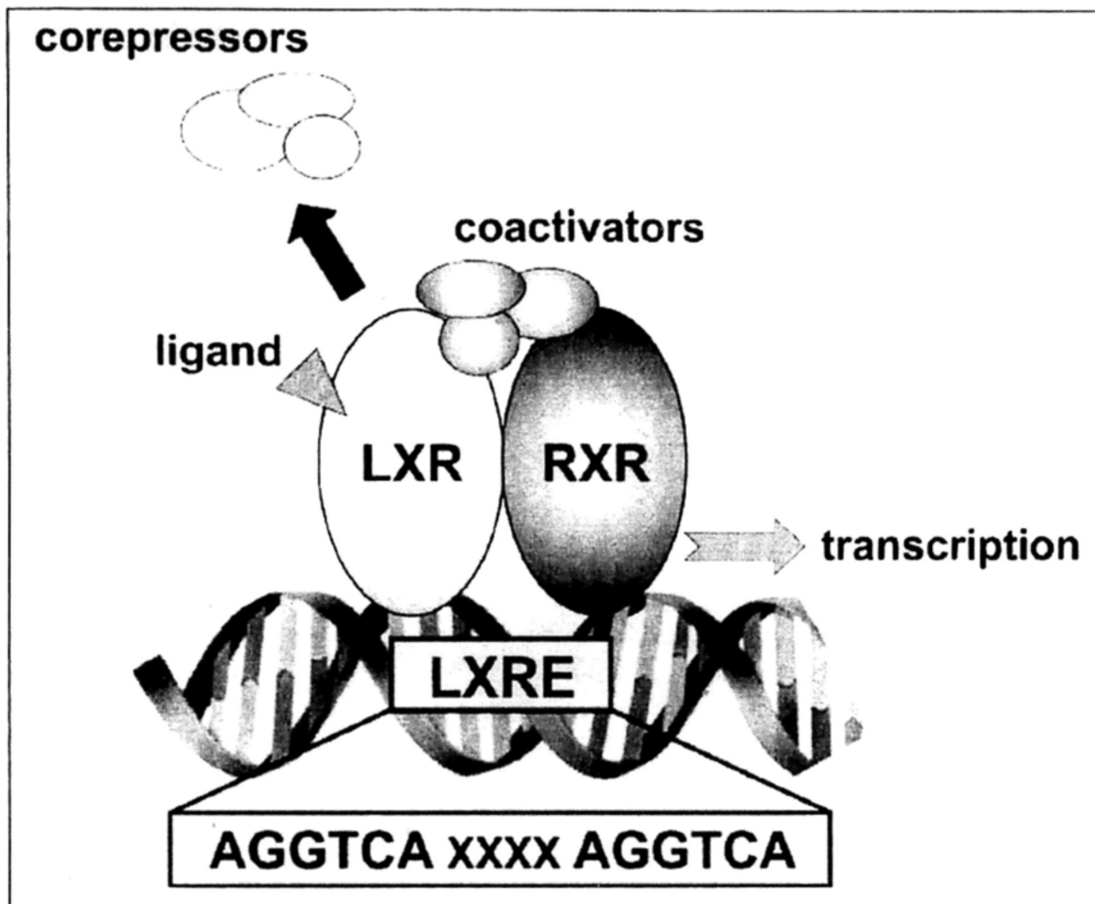


Figure 1.5

Mechanism of transcriptional regulation mediated by LXRs. RXR-retinoid X receptor, LXRE - LXR response element (Baranowski, 2008).

Identification of oxysterols as endogenous LXR ligands points to a role for these receptors in regulating expression of genes that are involved in cholesterol homeostasis (Figure 1.6). Indeed, the first reported gene directly regulated by LXRs is CYP7A1, the rate-limiting enzyme in hepatic bile acid synthesis (Lehmann et al., 1997). Also, LXRα deficient mice exhibit higher hepatic expression of SREBP-2 and several of its target genes including HMG-CoA synthase and reductase, farnesyl diphosphate synthase and squalene synthase (Peet et al., 1998). Further studies have demonstrated that LXR activation further enhances cholesterol excretion by increasing transcription of the ABCG5 and G8 (see 1.4.7 below) in the liver (Yu et al., 2003 & Repa et al., 2002). Both ABCG5 and ABCG8 expression is substantially increased upon LXR activation in murine intestine and in human enterocyte CaCo-2 cell line (Repa et al., 2002 & Duval et al., 2006 & Cavelier et al., 2006). In consequence administration of LXR agonists highly decreases intestinal cholesterol absorption in mice (Repa et al., 2002, Repa et al., 2000). Initially this effect was associated with up-regulated ABCA1 expression in enterocytes (Repa et al., 2000). In addition, it was reported that expression of NPC1L1 (see 1.4.6 below) is decreased by synthetic LXR agonists in cultured CaCo-2 cells as well as in murine intestine (Duval et al., 2006). Further more, the initial finding that LXRα plays a major role in maintaining hepatic cholesterol homeostasis suggested that LXRs may regulate "reverse cholesterol transport" (RCT) as well. In fact, subsequent studies have revealed that LXRs stimulate almost every aspect of this process. Cholesterol efflux from the cells is the first step in RCT process and is primarily mediated by ABCA1 and ABCG1 transporters. ABCA1 transfers both cholesterol and phospholipids from plasma membrane to lipid-free apolipoprotein A-I (apoA-I). This transporter is also important for the formation of nascent HDL particles in the liver. On the other hand, the function of ABCG1 is to transfer cholesterol to HDLs (Cavelier et al., 2006). It

was demonstrated that LXRα and LXRβ up-regulate ABCA1 and ABCG1 expression of rodent as well as human via functional LXREs found in their genes (Repa et al., 2000 &, Costet et al., 2000 & Sabol et al., 2005). In summary, LXRs have emerged as key sensors of intracellular sterol levels that trigger various adaptive mechanisms which include biliary cholesterol excretion, inhibition of intestinal absorption, suppression of cholesterol de novo synthesis and stimulation of reverse cholesterol transport (Baranowski, 2008).

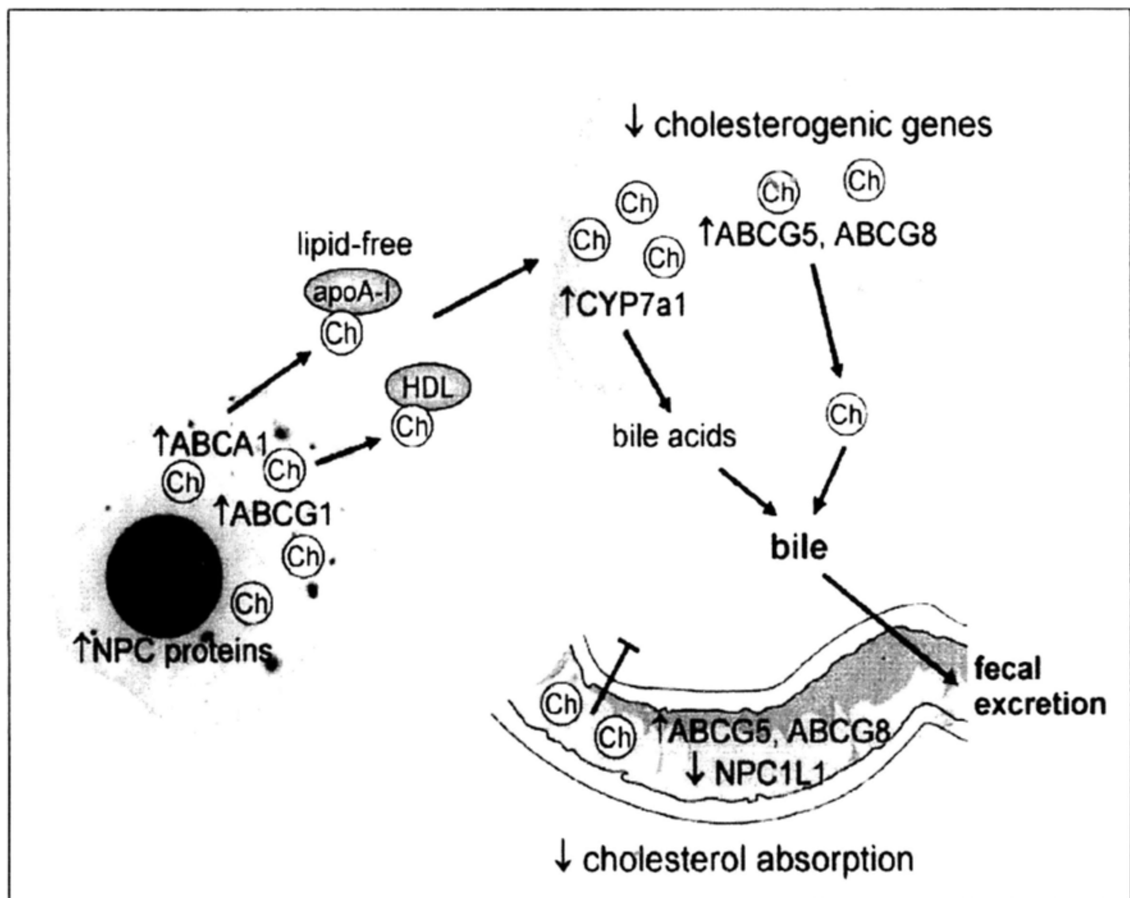


Figure 1.6

Role of LXRs in cholesterol transport and metabolism. LXR-regulated genes are indicated. Ch – cholesterol (Baranowski, 2008).

1.4.5 CYP7A1

The end products of cholesterol utilization are the bile acids, which are synthesized in the liver. Bile acids could be synthesized by two ways: 1) by the neutral or classical bile acid synthetic pathway regulated by CYP7A1; 2) by the alternative/acidic pathway regulated by CYP7B1 (Davis et al., 2002). However, bile acid biosynthetic pathway is commonly referred to the first one-the neutral or classic pathway (Swell et al., 1981 & Martin et al., 1993). From Figure 1.9, it can be found that CYP7A1 is a rate-limiting liver specific enzyme in bile acid classic pathway. CYP7A1 places a hydroxyl group onto the 7α position of the cholesterol within the endoplasmic reticulum. In human bile, the most abundant bile acids are chenodeoxycholic acid (45%) and cholic acid (31%) (Michael, 2010). These two are referred to as the primary bile acids. In the intestine, bacteria can convert the primary bile acids to the secondary bile acids, deoxycholate (from cholate) and lithocholate (from chenodeoxycholate). Both primary and secondary bile acids are reabsorbed by the intestines and delivered back to the liver via the portal circulation (Michael, 2010).

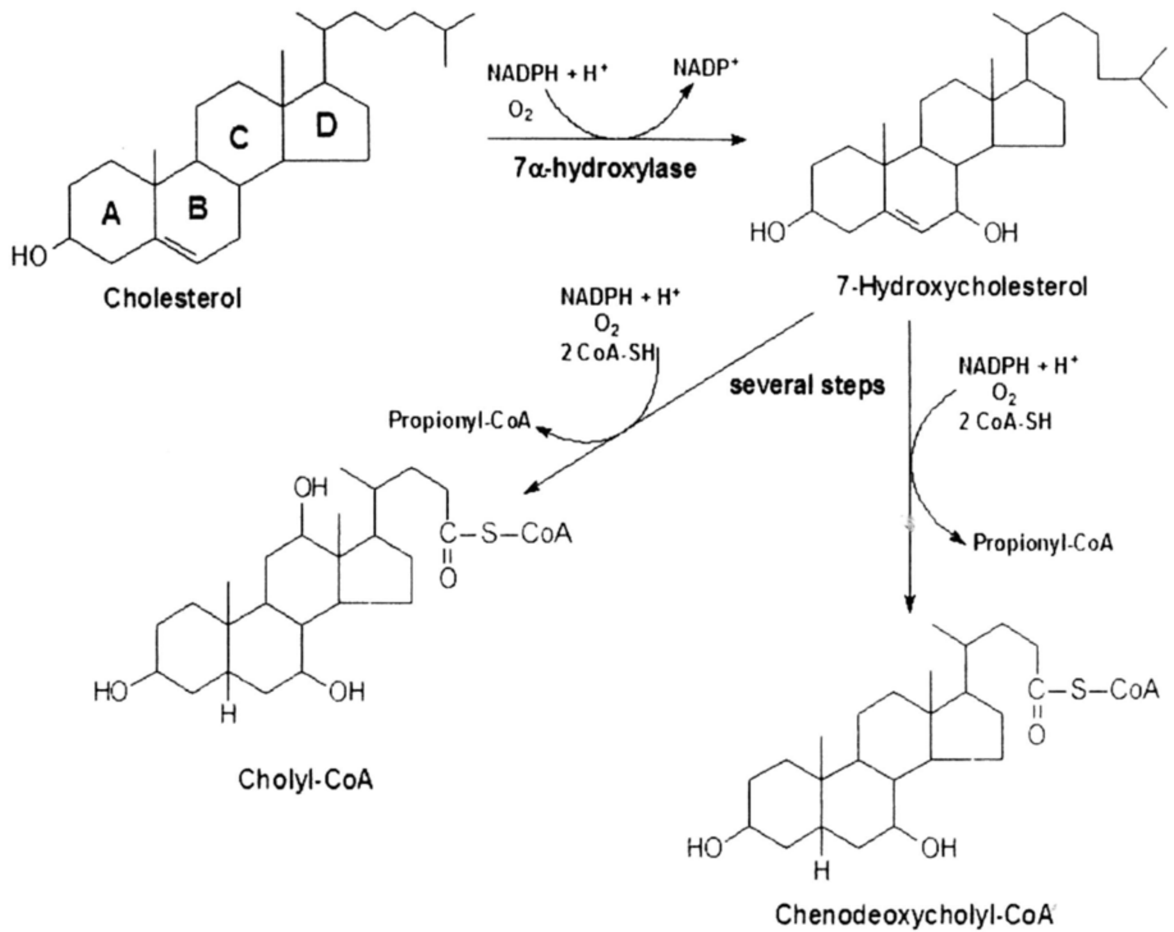


Figure 1.9

The classical bile acid synthetic pathway. Cholesterol-7α-hydroxylase (CYP7A1) is a rate-limiting liver specific enzyme in bile acid classic pathway (Michael, 2010).

1.4.6 NPC1L1

In humans, approximately 50% of intestinal cholesterol is absorbed, in which about two thirds is derived from the biliary cholesterol and the other one third is derived from the diet. Then the free cholesterol is transferred from the micelles to the surface of the brush border membrane of intestinal enterocytes by NPC1L1 (Davis et al., 2004). NPC1L1 is a protein localized in jejunal enterocytes that is important for intestinal cholesterol absorption, also, it can transport phytosterol from lumen into enterocytes (Tang et al., 2009 & Davis et al., 2004). However, phytosterols are poorly absorbed compared to cholesterol, and it is unknown whether the two sterol classes are taken up into intestinal enterocytes by the same mechanism. In rodents (mice, hamsters, and rats) NPC1L1 is primarily only found in the small intestine, while in humans and other species (monkeys, pigs and dogs). NPC1L1 is also expressed in the liver, but its highest expression in these species is also in the small intestine (Telford et al., 2007 & Zúñiga et al., 2008 & Valasek et al., 2008)

Since cholesterol synthesis and cellular uptake is tightly regulated, blocking intestinal cholesterol uptake by NPC1L1 deficiency or inhibition may affect the intestinal and hepatic expression of genes involved in sterol metabolism. NPC1L1 has been shown to be down-regulated by cholesterol feeding in mice and up-regulated by ezetimibe treatment in pigs (Davis et al., 2004 & Telford et al., 2007). Increased intracellular cholesterol levels caused by cholesterol containing diets or ACAT2 deficiency is thought to down regulate NPC1L1 (Davis et al., 2004, Temel et al., 2005 & Alrefai et al., 2007). NPC1L1 has also been shown to be regulated in-vivo and in-vitro by a variety of other mechanisms, including peroxisome proliferator activated receptor (PPAR) alpha and delta agonists (Valasek et al., 2007& van der Veen et al., 2005), LXR agonists (Valasek et al., 2007), RXR agonists (Lalloyer et al., 2006), long chain fatty acids and high triglyceride diets (de

Vogel-van den Bosch et al., 2008 & Mathur et al., 2007), and diabetes (Lally et al., 2006), but the precise mechanisms of NPC1L1 regulation are not known and are being actively investigated presently.

1.4.7 ABCG5/8

As we already know that NPC1L1 is a cholesterol uptake transporter, while the ABCG5 and ABCG8 can be regarded as cholesterol efflux transporters (Berge et al., 2000 & Lee et al., 2001 & Lu et al., 2001). These three molecules appear to be key players in the control of the cholesterol absorption from the intestinal lumen.

ABC transporters are a large family of proteins which mediate transport of a wide variety of substrates across different cellular membranes (out of bacterial and eukaryotic cells), processes driven by the hydrolysis of adenosine-triphosphate (ATP) (Dean et al., 2001). ABCG5 and ABCG8, which function as a heterodimeric transporter, are critical for the control of sterol absorption (Graf et al., 2003). These proteins are localized at the canalicular membrane of hepatocytes and at the brush border of enterocytes. ABCG5 was found to be localized adjacent to ABCG8 on chromosome 2p21 and to be coordinately regulated with ABCG8 through common regulatory elements (such as the nuclear receptor LXR), resulting in similar tissue- and cell-specific expression patterns (Berge et al., 2000). Dietary cholesterol leads to an increase in the expression of the ABCG5 and ABCG8 transporters in mice which is controlled by the activation of LXRs (Berge et al., 2000 and Repa et al., 2002). Biliary cholesterol concentrations were extremely low in ABCG5/ABCG8 (−/−) knockout mice when compared with wild-type animals (Yu et al., 2002).

Phytosterols (such as sitosterol and campesterol) are not synthesized in humans and are derived entirely from the diet. Once absorbed by intestinal cells, ABCG5/ABCG8 transporters normally pump these potentially atherogenic

phytosterols back into the intestinal lumen, normally resulting in <5% net absorption (Bays et al., 2003). Mutations in the genes encoding ABCG5 and ABCG8 transporters cause sitosterolemia (Berge et al., 2000 & Lee et al., 2001 & Lu et al., 2001), which is characterized by the accumulation of plant sterols in blood and other tissues as a result of their enhanced absorption from the intestines and decreased removal in bile.

1.4.8 ACAT

ACAT genes, ACAT1 and ACAT2, have been identified that encode 2 proteins responsible for intracellular cholesterol esterification. ACAT2 expression is predominant in the liver and intestine and ACAT1 exists in most tissues of the body, but its level of expression in the mouse small intestine is very low (Anderson et al., 1998 & Oelkers et al., 1998 & Meiner et al., 1997).

ACAT2 is highly specific for cholesterol and does not esterify plant sterols. Intestinal ACAT2 is the primary enzyme responsible for the intracellular cholesterol esterification. In humans, ACAT2 plays a crucial role in the absorption of cholesterol in the small intestine, before cholesterol is incorporated into CM and transported primarily as CE into the body (Largis et al., 1989). The metabolism of chylomicrons in the circulation leads to the formation of remnant lipoproteins that retain the CE, which is eventually delivered to the hepatocytes, and hydrolysis of the cholesteryl ester by cholesteryl ester hydrolase (CEH) follows uptake and some of the resulting free cholesterol becomes available for re-esterification by ACAT2 (Rudel et al., 2005) (Figure 1.10). In the liver, this enzyme is partially responsible for the assembly of VLDL prior to secretion into the blood (Drevon et al., 1980). TG-rich VLDL derived from the liver is transformed into cholesterol-rich LDL after the removal of their TG by peripheral tissues (Rudel et al., 2005). The free

cholesterol is either effluxed out of the cell or, in situations in which cholesterol is excessive, such as during developing atherosclerosis, it may be resynthesized by ACAT1 and stored in lipid droplets. The process of synthesis and hydrolysis of cholesteryl ester is dynamic. When many of these droplets are formed, the macrophages become known as foam cells. Therefore, ACAT1 has a key role in the development of early atherosclerotic lesions in that it generates the cholesteryl ester of the foam cell, albeit in response to excessive cholesterol availability within the cell (Rudel et al., 2005). Cholesterol that is effluxed from cells such as the macrophage appears in the circulating HDL, where it can be esterified by the plasma enzyme lecithin-cholesterol acyltransferase (LCAT) for the eventual return of the cholesteryl ester to the liver. Therefore, the role of ACAT1 is to maintain the appropriate FC levels inside cells, presumably in response to the needs of membrane function (Rudel et al., 2005). Inhibition of ACAT activity therefore lowers the plasma cholesterol level by decreasing cholesterol absorption in the intestine and VLDL production in the liver (Chen et al., 2009).

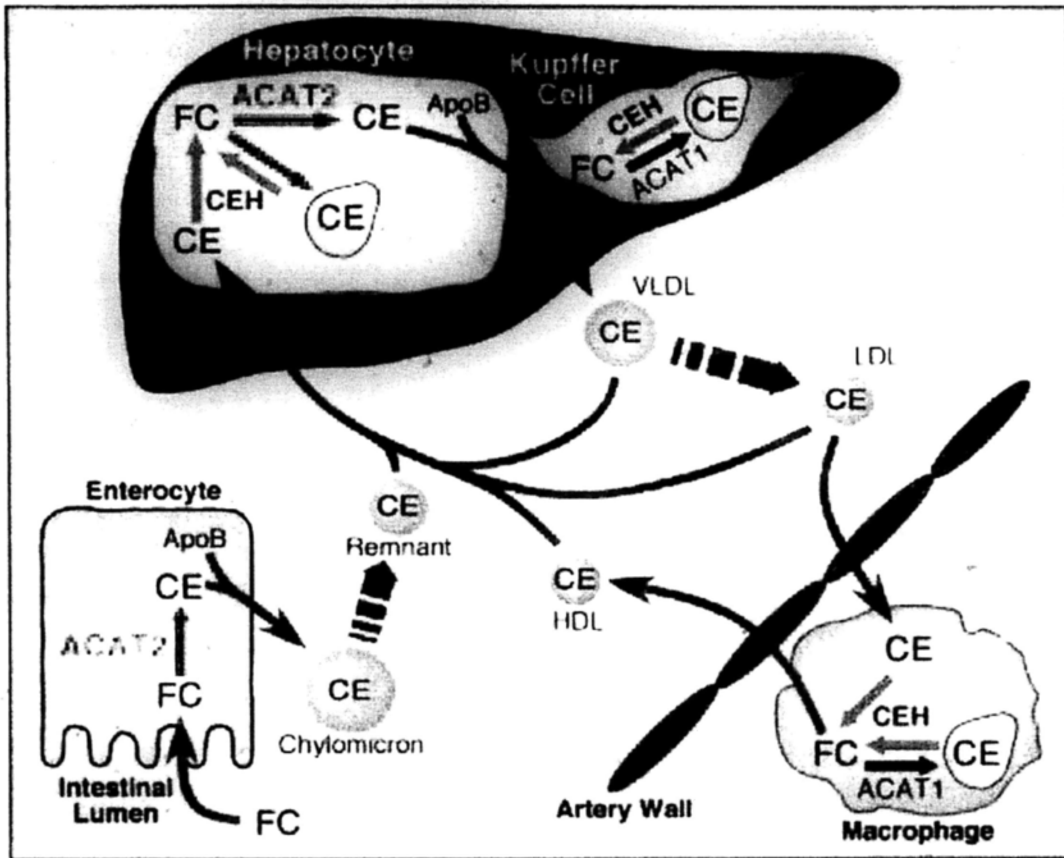


Figure 1.10

Diagram of the separate roles of ACAT1 and ACAT2 in cholesterol metabolism in the liver, enterocyte, and macrophages within the artery wall (Rudel et al., 2005).

1.4.9 MTP

MTP is a heterodimeric lipid transfer protein that catalyzes the transport of triglyceride, cholesteryl ester, and phosphatidylcholine between membranes (Jamil et al., 1996). MTP activity has been characterized mainly in the liver and the small intestine of the bovine and rat (Wetterau et al., 1986), and also the specific expression of the large subunit is more recently demonstrated in humans (Shoulders et al., 1993), mouse (Nakamuta et al., 1996) and hamsters (Lin et al., 1994).

Lipoproteins are assembled in the endoplasmic reticulum, matured in the Golgi, and secreted by cells. Their biosynthesis depends on two proteins: apolipoprotein B (apoB) and MTP (Hussain et al., 2003). Recent findings have shown that MTP could interact with nascent apoB, the main apolipoprotein component of VLDL particles, acting both as a chaperone protein and as a source of TG (Bakillah et al., 2003). ApoB is regarded as a structural protein. MTP is an essential chaperone for the assembly of apoB lipoproteins (Hussain et al., 2002 & Hussain et al., 2003 & Hussain et al., 2008). Several arguments support the hypothesis that MTP activity could be involved in the transfer of TG from their site of synthesis on the membrane of the endoplasmic reticulum to the site of VLDL assembly, in the lumen of the endoplasmic reticulum (Graulet et al., 2004).

A reduction of MTP activity has also been correlated to the development of alcoholic fatty liver in rats (Sugimoto et al., 2002). Besides, in patients suffering from genetic abetalipoproteinemia, the defect in hepatic and intestinal production of apo B-containing lipoproteins has been correlated to the lack of efficient MTP activity (Berriot-Varoqueaux et al., 2000). Moreover, the effects of nutritional factors on MTP activity have been relatively less studied. In rodents, hepatic MTP activity increased with increasing dietary fats intake (Lin et al., 1994 & Bennett et al., 1995). Additionally, it has been shown that cholesterol (Bennett et al., 1996; Billett et al.,

2000) or a sucrose-rich diet also increased hepatic MTP mRNA level in hamsters (Lin et al., 1994).

1.5 The effect of individual dietary food on plasma lipoproteins

1.5.1 Polyphenols

Polyphenols are a group of compounds found in plants, characterized by the presence of more than one phenol unit or building block per molecule. Polyphenols are generally divided into hydrolyzable tannins (gallic acid esters of glucose and other sugars) and phenylpropanoids, such as lignins, flavonoids, and condensed tannins (Figure1.11). For example, the polyphenols present in grape and its seed are mainly hydroxycinnamic acid, flavonols, anthocyanins, catechins, and proanthocyanidins (Morel-Salmi et al., 2006). Polyphenols and related compounds can be found in every plant species. They can be found in fruits, vegetables, coffee, tea, chocolate, and soy (Scalbert et al., 2002). Once ingested, polyphenols have several possible fates, including absorption in the small intestine or colon, and/or excretion in the feces or urine. The site and rate of absorption depend on their chemical structure, conjugation of other phenolics, degree of glycosylation/acylation, degree of polymerization, molecular size, and solubility (Bravo, 1998 & Scalbert et al., 2002). In the small intestine, polyphenols can enter the mucosa through passive diffusion. In the colon, polyphenols are initially digested into smaller phenolic structures by gut microflora (Bravo, 1998). After this initial digestion finished, the polyphenols and their metabolites might be absorbed and transported to the liver. (Bravo, 1998).

Polyphenols have generated a great amount of scientific research in vivo and in vitro due to their antioxidant capabilities. For many years, red wine has been thought to possess beneficial effects on cardiovascular health. This relation is clear in the

French Paradox phenomenon as well as in the Mediterranean diet. The French Paradox is defined as a low incidence of coronary heart disease (CHD) while consuming a diet high in saturated fat (De Logngeril et al., 1996). The Mediterranean diet, rich in fruits and wine, is shown to protect against the occurrence of cardiovascular disease (De Logngeril et al., 1996, Hertog et al., 1995). Furthermore, the inclusion of fruits and vegetables in the diet may help reverse hyperlipidemia, alter the atherogenicity of the LDL particle (Lampe et al., 1999), and protect the cholesterol from oxidation in LDL (Brouillard et al., 1997). Besides, favorable modification of lipoproteins by decreasing the LDL-C/HDL-C ratio and oxidation of LDL-C has also been claimed to be responsible for the reduced risk of coronary heart disease associated with moderate consumption of red wine (de Gaetano et al., 2003/2004).

Zern et al (2005) have summarized the effect of dietary polyphenols on cholesterol and related lipoproteins: 1) Reduction in cholesterol absorption will result in decreased delivery of cholesterol to the liver by chylomicron remnants, which in turn will 2) up-regulate hepatic LDLR mRNA abundance to compensate for less substrate availability and induce reductions in plasma cholesterol; 3) Polyphenols affect apo B secretion rates, MTP, and ACAT2 activities, resulting in a modified VLDL particle; 4) Reductions in plasma TG are shown as a result of lowered MTP activity, possibly increased LPL activity, further alter the delipidation cascade, and yielding less LDL in circulation.

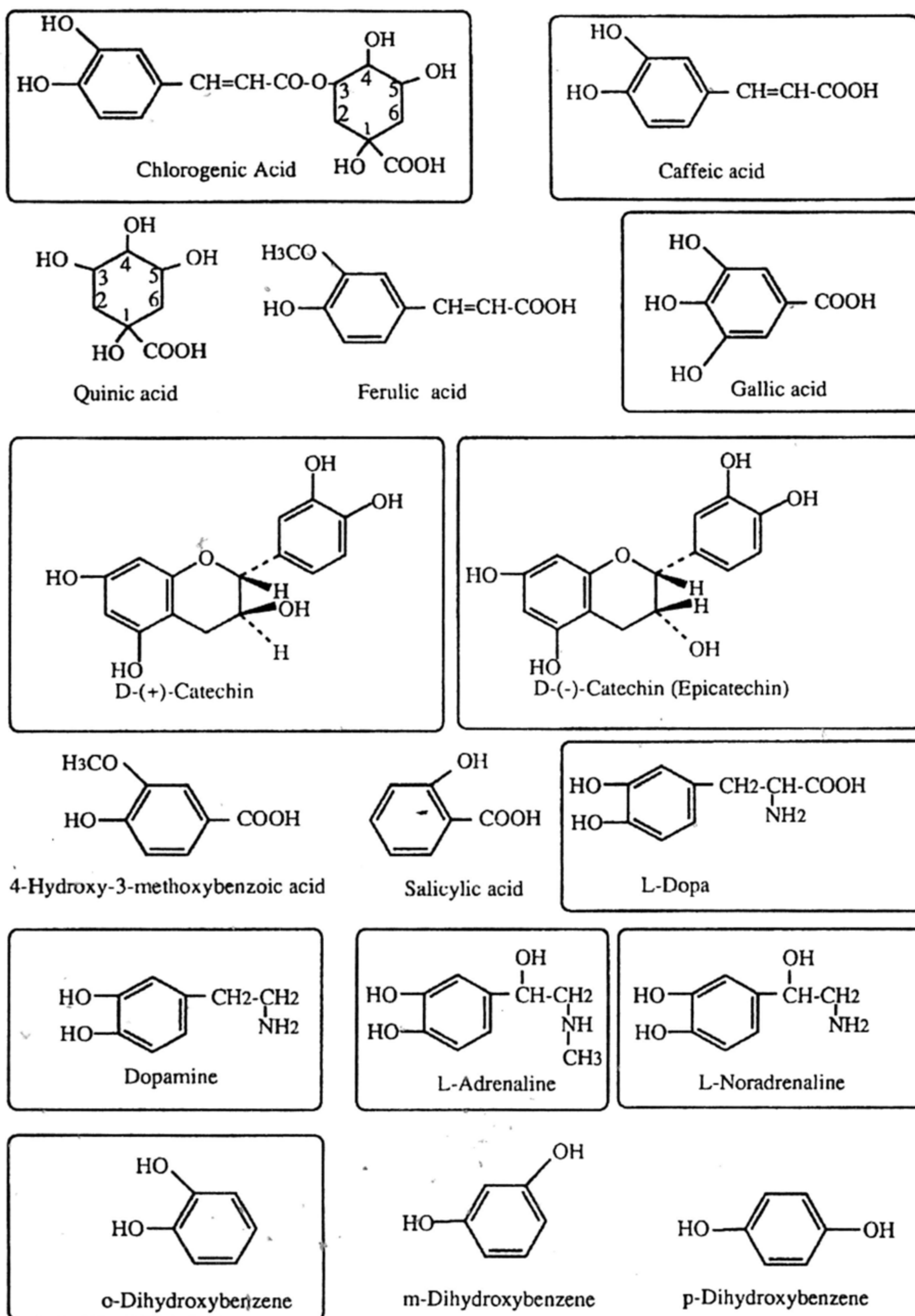


Figure 1.11

Structures of polyphenols and their related compounds (Iwahashi, 2000)

1.5.2 Sterols

1.5.2.1 Dietary cholesterol

Extensive studies have shown that dietary cholesterol increase plasma cholesterol levels and the risk of coronary heart disease. Cholesterol is rich in animal product, such as eggs, meat, especially organs, such as brain, kidney and liver; also it's rich in animal fat and seafood. Daily dietary cholesterol intake is recommended to be less than 300 mg/day, however, in developed and some developing countries, cholesterol consumption is much more than that. In a typical western diet consumed by an average adult, nearly 300-500 mg dietary cholesterol and 900-1200 mg biliary cholesterol totally 1200-1700 mg cholesterol enters the small intestine lumen each day. Then, about half of the cholesterol is excreted in feces, the remainder is absorbed into the intestinal cells, then esterified and packaged into chylomicrons. As CMs leave the intestine, their cholesterol is transported through the lymphatic system to the blood and the liver. Most dietary cholesterol exists in the form of the free sterol, with only 10% to 15% existing as the cholesteryl ester. Only non-esterified cholesterol could be incorporated into bile acid micelles and absorbed by enterocytes, so cholesteryl ester must be hydrolyzed by cholesterol esterase to release free cholesterol for absorption.

1.5.2.2 Phytosterols

Plant sterols and their saturated derivatives, stanols, are a group of cholesterol analogues with different side-chain configurations (Figure 1.12). The principal sterols are β-sitosterol, campesterol, and stigmasterol. A major application for these phytosterols is their addition into spreads and vegetable oils (functional margarine, butter, and cooking oils) (Chen et al., 2008).

Phytosterols are poorly absorbed in the intestine, only about 5% is absorbed

(Temel et al., 2003). A number of studies in humans have indicated that phytosterols have the cholesterol-lowering activity. It is estimated that the phytosterol intake in humans can reach 160−360 mg/day (Ling et al., 1995), and it has been suggested that the daily consumption of 2 g of phytosterols can effectively lower the cholesterol by 9−14% in humans with little or no effect on HDL-C and TG levels (Law, 2000). Another study showed that when children with severe familial hypercholesterolemia (TC < 370 mg/dL) were given 2 g of sitosterol three times a day for 3 months followed by 0.5 g sitostanol three times a day for an additional 7 months, LDL-C was reduced by 20% and there was a significant increase in fecal neutral sterols excretion, indicating inhibition of intestinal cholesterol absorption (Becker et al., 1993). Also, it has been shown that plant sterol esters and stanol esters in margarine are equally effective in lowering TC and LDL-C in normocholesterolemic and mildly hypercholesterolemic volunteers (Weststrate et al., 1998).

The cholesterol-lowering activity of phytosterols is also clear in animal studies. A 5:1 ratio of phytosterols/cholesterol was found to effectively block cholesterol absorption in gerbil when the dietary cholesterol was moderate (Hayes et al., 2002). A study of mice found that dietary plant sterols and stanols inhibited cholesterol absorption in the intestinal lumen, but that the effect was independent of LXR (Plo¨sch et al., 2006). Dietary phytosterol intake has been shown to inhibit cholesterol absorption and lower plasma LDL-C in guinea pigs (Ramjiganesh et al., 2001) and to prevent the development of aortic foam cells in hamsters (Ntanios et al., 2003).

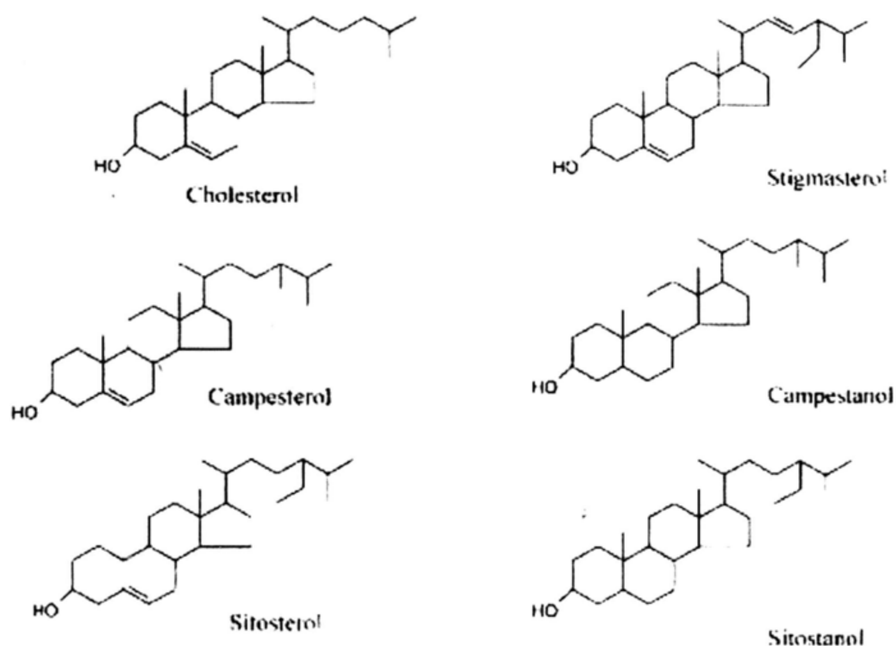


Figure 1.12

Structure of selected phytosterols (Chen et al., 2008).

1.5.3 Saturated fatty acids

Saturated fatty acids (SFA) are a group of long-chain carboxylic acids that usually have a carbon number between 12 and 24 no double bonds. SFAs are mainly from animal products and certain vegetable oils, including coconut oil, palm oil and cocoa butter. Dietary SAFs are hypercholesterolemic relative to monounsaturated and polyunsaturated fatty acids.

For years, abundant studies have been conducted in animal models to elucidate the mechanisms by which different types of fatty acids modulate plasma cholesterol concentrations. SFAs and trans-fatty acids are undesirable on plasma lipids, whereas polyunsaturated fatty acids of the (n-6) family and monounsaturated fatty acids decrease plasma LDL-C concentrations. Among the SFAs, stearic acid (18:0) appears to have a neutral effect on LDL-C, while lauric (12:0), myristic (14:0), and palmitic (16:0) acids are considered to be hypercholesterolemic. SFAs increase plasma LDL-C by increasing the formation of LDL in the plasma compartment and by

decreasing LDL turnover (Fernandez et al., 2005). Furthermore, the increase in plasma LDL-C concentrations associated with consumption of saturated vegetable oils and fats is largely explained by a decrease in hepatic LDL receptor activity and an increase in the LDL-C production rate (Nicolosi, 1997). Animal data for the effects of individual fatty acids on plasma LDL-C concentrations and metabolism suggest that caproic acid (6:0), caprylic acid (8:0), and capric acid (10:0) are neutral with respect to their effect on LDL-C. In contrast, lauric acid, myristic acid, and palmitic acid are equally effective in raising LDL-C level by reducing hepatic LDL receptor activity and increasing the LDL-C production rate, apparently via modulation of sterol O-acyltransferase activity. Stearic acid also appears to be neutral as it has no effect on LDL-C level (Nicolosi, 1997).

Chapter 2

Effect of Grape Seed Proanthocyanidin on Lipoprotein Metabolism in Hamsters

2.1 Introduction

Grape seed proanthocyanidins (GSP) refer to a group of procyanidins consisting of a mixture of dimers and oligomers of catechin and epicatechin and their gallic acid esters. GSP has been a subject of the extensive investigations for its various biological activities. It has been reported that GSP is anti-atherosclerosis (Frederiksen et al., 2007; Polagruto et al., 2007), anti-carcinogenesis (Kaur et al., 2008), hypotensive (Peng et al., 2005), anti-lipogenesis(Moreno et al., 2003), antioxidant (Cetin et al., 2008; Sakurai et al., 2010), and anti-hyperglycemic (Hwang et al., 2009). GSP has been also shown to be hypocholesterolemic in both humans and animals (Frederiksen et al., 2007; Preuss et al., 2000), although the underlying mechanism remains poorly understood.

2.2 Objective

Despite some research on GSP, little is known of how it interacts with the genes and proteins involved in lipoprotein metabolism *in vivo*. The present study was therefore undertaken to (i) characterize interaction of dietary GSP with SREBP-2, LXRα, HMG-CoA-R, LDLR, and CYP7A1; and (ii) investigate the effect of dietary GSP on fecal excretion of individual neutral and acidic sterols in hamsters.

2.3 Materials and methods

2.3.1 GSP Analysis

GSP was obtained from Jianfeng Natural Product Study Ltd, Co., Tianjin, China. Individual components in GSP were determined using high performance liquid chromatography (HPLC). In brief, GSP sample was injected into an HPLC column (YMC C_{18}, 5 μm, 250×4.6 mm ID) and quantified on a Shimadzu LC-10AT HPLC system equipped with a UV detector at 280 nm. The elution profile was programmed at a flow rate of 1 mL/min, while the gradient mobile phase composed of solvent A (2% acetic acid) and solvent B (acetonitrile:water:acetic acid; 80:19.6:0.4; vol/vol/vol). The ratio of A to B was programmed: 0-3 min, 100:0; 3-6 min; changed to 96:4; 6-15 min, changed to 90:10; 15-30 min, changed to 85:15; 30-50 min; changed to 77-23; 50-60 min, changed to 75:25; 60-66 min, changed to 70:30; 66-80 min; changed to 50:50; 80-83 min, changed to 20:80; 83-85 min, changed to 100:0 and then was held for additional 5 min (Figure 2.1). On the basis of peak areas, GSP contained 6.04% dimmer procyanidins (peaks 1 and 2), 26.50% catechin (peak 3), 10.53% procyanidin B2 (peak 4), 31.01% epicatechin (peak 5), 10.23% dimmers esterified gallic acid (peaks 6 and 9), 4.10% trimers esterfied with gallic acid (peaks 7 and 8), and 3.63% trimers esterified with gallic acid (peak 10).

2.3.2 Diets

Three diets were prepared as previously described (Lam et al., 2008). The control diet contained the following ingredients in proportion (g/kg diet): cornstarch, 508; casein, 242; lard, 50; sucrose, 119; mineral mix AIN-76, 40; viamin mix AIN-76A, 20; DL-methionine, 1; cholesterol, 1 (Table 2.1). The two experimental diets were prepared by adding 0.5% and 1.0% GSP (w/w) into the

control diet, respectively. The powered diets were mixed with a gelatin solution (20 g/L) in a ratio of 200 g diet per liter of solution. Once the gelatin has set, the diets were cut into pieces of approximately 10 g cubes and stored frozen at -20°C.

2.3.3 Animals

Thirty-three (113±6 g) male adult Golden Syrian hamsters (Mesocricetus auratus) were randomly divided into three groups (n=11) and housed individually in wire-bottomed cages at 23°C in a 12-hour light-dark cycle animal room. Before the experiment, all the hamsters were allowed to stabilize by being fed a 0.1% cholesterol diet for two weeks (control diet). During the following 6 weeks, one group were continued to be fed the control diet and the other two groups were fed one of the two experimental diets containing 0.5% GSP or 1.0% GSP. During the study, food was given daily, any uneaten food was discarded, the amount of food consumed was measured each day, and total fecal output was collected. The hamsters were free to access to food and distilled water, and were weighed weekly. Blood (0.5 ml) was bled from the retro-orbital sinus into a heparinized capillary tube under light anesthetization, using a mixture of ketamine, xylazine and saline (v/v/v, 4:1:5), at the end of weeks 0, 3 and 6 after food deprivation for 14 hr. The blood was centrifuged at 1000 × g for 10 min and the plasma was collected and stored at -20°C until analysis. After a 3-day recovery, all hamsters were sacrificed by carbon dioxide suffocation. Blood was collected from abdominal artery into a vacuum heparinized tube and centrifuged at 1000 × g for 10 minutes, the plasma was collected and stored at -20°C until analysis. The liver, heart, and kidney were removed, washed in saline, weighed, flash frozen in liquid nitrogen and stored at -80°C until analysis. Experiments were conducted following the approval and guidelines set by the Animal Experimental Ethical Committee, The Chinese

University of Hong Kong.

2.3.4 Determination of serum lipoproteins

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

2.3.5 Measurement of organ cholesterol

Cholesterol in organs was determined using a method as previously described (Lam et al., 2008). In brief, organ sample (300 mg) was cleaned of adventitial tissue and washed in saline solution. Total lipids were extracted with addition of 0.2 mg stigmastanol (1.0 mg for the liver sample) as an internal standard using chloroform-methanol (2:1, v/v). The lipid extracts were then saponified with 6 mL of 1 mol/L NaOH in 90% ethanol at 90°C for 1 h, and the non-saponified substances including cholesterol were converted to their trimethylsilyl (TMS)-ether derivatives by a commercial TMS reagent (Sigma-Sil-A, Sigma, St. Louis, MO, USA). The analysis of cholesterol TMS-ether derivative was performed in a fused silica capillary column (SAC^{TM}-5, 30 m × 0.25 mm, i.d.; Supelco, Inc., Bellefonte, PA, USA) using a Shimadzu GC-14 B GLC equipped with a flame ionization detector as previously described (Lam et al., 2008).

2.3.6 Determination of fecal neutral and acidic sterols

Individual fecal neutral and acidic sterols were quantified as previously

described (Lam et al., 2008; Chan et al., 1999). Total fecal sample from each hamster was freeze-dried, ground and well mixed. In brief, stigmasterol (0.3 mg) as an internal standard for neutral sterols was added to a fecal sample (300 mg). The sample was saponified using 9 mL of 1 mol/L NaOH in 90% ethanol containing 0.3 mg hyodeoxycholic acid as an internal standard for acidic sterols (Sigma, St. Louis, MO, USA). The total neutral sterols were extracted using 8 mL of cyclohexane and were then converted to their corresponding TMS-ether derivatives for GLC analysis.

The remaining aqueous layer was saved for the analysis of acidic sterols. In brief, 1 ml of 10 M NaOH was added into the remaining aqueous layer and heated at 120°C for 3 h. After cooling down, 1 ml of distilled water and 3 ml of 3 N HCl were added followed by extraction using 7 ml of diethyl ether twice. The diethyl ether layers were then pooled followed by adding 2 ml of methanol, 2 ml of dimethoxypropane and 40 μl of concentrated HCl (12 mol/l). 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.

2.3.7 Western blotting analyses

Total liver proteins were extracted according to the method described previously by Vaziri and Liang with some modification (Vaziri et al., 1996). Briefly, the liver sample was homogenized in 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). The extract was centrifuged at 13,000 × g for 15 min at 4°C and the supernatant was collected (total protein). The total protein was centrifuged at 126,000 × g for 60 min at 4°C. The pellet (both membrane and nuclear proteins) was re-suspended in the same

homogenizing buffer. Membrane and nuclear proteins were separated by electrophoresis in a 7% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using a semi-dry transfer system. Membranes were then blocked in 5% nonfat milk Tris-buffered saline with Tween-20 for 1 hour and overnight at 4°C in the same solution containing anti-LDLR antibody (Santa Cruz Biotechnology, Inc., California, USA), anti-HMG-CoA-R (Upstate USA Inc., Lake Placid, NY, USA), anti-CYP7A1 (Santa Cruz Biotechnology, Inc., California, USA), or anti-SREBP-2 antibody (Santa Cruz Biotechnology) (Chan et al, 1999). The membrane was then incubated for one hour at 4°C in diluted horseradish peroxidase-linked Goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc. California, USA), donkey anti-rabbit IgG (Santa Cruz Biotechnology, Inc. California, USA) or goat anti-mouse IgG (Calbiochem, EMD Chemicals, Inc., San Diego, CA, USA). Then, membranes were developed with ECL enhanced chemiluminescence agent (Santa Cruz Biotechnology, Inc., California, USA) and subjected to autoradiography on SuperRX medical X-ray film (Fuji, Tokyo, Japan). Densitometry was quantified using the BioRad Quantity one® software (BioRad Laboratories, Hercules, USA). Data on abundance of SREBP-2, LDLR, HMG-CoA R, LXRα and CYP7A1 were normalized with β-tubulin (Santa Cruz Biotechnology, Inc., California, USA) (Chan et al., 1999).

2.3.8 Real time PCR analyses

Total mRNA levels for liver SREBP-2, LDLR, HMG-CoA-R, LXRα and CYP7A1 were quantified as previously described (Lam et al., 2008). Briefly, total liver mRNA was extracted and isolated using Trizol Reagent (Invitrogen, Carslbad, CA, USA) according to the manufacturer's instruction. Total RNA was converted to complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City,

CA, USA). Real-time PCR analysis was carried out on a Fast Real-time PCR System 7500 (Applied Biosystems). All primers and TaqMan probes used for real-time PCR for these genes were listed in Table 2.2 except for those of LXRα, which was purchased from Applied Biosystems). Real-time PCR was performed using a TaqMan Fast Universal PCR Master Mix (Applied Biosystems). The expressions of target genes were normalized with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.3.9 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, USA). Significance was defined as *P*-value less than 0.05.

2.4 Results

2.4.1 Effect of GSP on body weight and food intake

The body weight gain and organ weight were similar among the three groups (Table 2.3). GSP did not affect the weight gain and organ weights compared with the control hamsters. Food intake ranged 11.9-12.0 g/day/hamster among the three groups. No significant difference in food intake was seen between the control and the two experiment diet groups.

2.4.2 Effect of GSP on serum TC, TG and HDL-C

No differences in TC, HDL-C, non-HDL-C, ratio of non-HDL-C/HDL-C and TG were seen at the beginning of the experiment (Table 2.4). At the end of week 3, there was a decreasing trend in TC, non-HDL-C and TG in response to the increasing amount of GSP in diet. TG-lowering activity of GSP was dos-dependent

at the end of week 3. At the end of week 6, plasma TC in the two experimental groups decreased in a dose-dependent manner. A significant decreasing trend in plasma non-HDL-C and TG in response to the increasing amount of GSP was observed. To be specific, plasma TC in 0.5% GSP and 1.0% GSP groups was reduced by 4% and 16%, while non-HDL-C was reduced by 5% and 22%, respectively. Similarly, plasma TG in 0.5% GSP and 1.0% GSP was reduced by 12% and 30%, respectively, compared with the control at the end of week 6.

2.4.3 Effect of GSP on organ cholesterol

Total cholesterol in the liver decreased significantly in the 1.0% GSP group compared with the control group (Figure 2.2). No difference in hepatic cholesterol level was seen between the control and 0.5% GSP group. There were no significant differences in the cholesterol level of heart and adipose among the three groups.

2.4.4 Effect of GSP on fecal neutral and acidic sterols

Supplementation of GSP into diets caused greater fecal excretion of acidic sterols compared with the control diet group in a dose-dependent manner (Table 2.5). The major acidic sterols affected included lithocholic acid, chenodeoxycholic acid, cholic acid and deoxycholic acid. However, no significant difference in fecal excretion of neutral sterols was seen among the control and the two GSP groups.

2.4.5 Effect of GSP on cholesterol balance

Total intake of cholesterol was compared with its excretion of total neutral and acidic sterols (Table 2.6). Net cholesterol equivalent retained was calculated by difference between intake and excretion of both neutral and acidic sterols. It was found that net cholesterol retention was the most in the control followed by 0.5%

GSP and 1.0% GSP in a decreasing order. The apparent cholesterol absorption was calculated in an equation [(cholesterol intake - excretion of neutral and acidic sterols)/cholesterol intake]. It proved that GSP decreased cholesterol absorption in a dose-dependent manner.

2.4.6 Effect of GSP on immunoblot and mRNA analyses of SREBP-2, LDLR, HMG-CoA-R, CYP7A1 and LXRα

The western blot and RT-PCR analyses demonstrated that supplementation of GSP into diets had no significant effect on hepatic SREBP-2, LDLR in hamsters (Figure 2.3). Although the immunoblot analysis showed that GSP had no effect on the protein mass of HMG-CoA-R, the real tim-PCR analysis demonstrated that GSP could up-regulate the production of mRNA HMG-CoA-R. Results demonstrated that GSP had no effect on the protein level of LXRα. However, mRNA LXRα was down-regulated greater in hamsters fed the 1.0% GSP (Figure 2.4). In contrast, GSP up-regulated not only the protein level but also the mRNA level of CYP7A1.

Table 2.1 Composition (%) of the control and two experimental diets containing 0.5% grape seed proanthocyanidin (0.5% GSP) and 1.0% grape seed proanthocyanidin (1.0% GSP).

Component	Control	0.5% GSP	1.0% GSP
Cornstarch	50.8	50.8	50.8
Casein	24.2	24.2	24.2
Sucrose	11.9	11.9	11.9
Lard	5	5	5
Mineral mix	4	4	4
Vitamin mix	2	2	2
Gelatin	2	2	2
DL-Methionine	0.1	0.1	0.1
Cholesterol	0.1	0.1	0.1
GSP	--	0.5	1.0

Table 2.2 List of oligonucleotide primers and probes used to amplify mRNA by real-time PCR

Gene	Accession number	Forward primer 5′ → 3′	Reverse primer 5′ →3′	TaqMan probe 5′
GAPDH	**DQ403055**	GAACATCATCCCTGCATCCA	CCAGTG AGCTTCCCGTTCA	CTTGCCCACAGCCTTGG CAGC
CYP7A1	**L04690**	GGTAGTGTGCTGTTGTATATGGGTTA	ACAGCCCAGGTATGG AATCAAC	CACCTGCTTTCCTTCTCC
HMG-CoA-R	**X00494**	CGAAGGGTTTGCAGTGATAAAGGA	GCCATAGTCACATGAAGCTTCTGTA	ACGTGCGAATCTGCT
LDLR	**M94387**	GCCGGGACTGGTCAG ATG	ACAGCCACCATTGTTGTCCA	GCACTCATTGGTCCT GCAGTCCTT
SREBP-2	**U12330**	GGACTTGGTCATGGGAACAGATG	TGTAATCAATGGCCTTCCTCAGAAC	CCAAGATGCACAA ATC

Table 2.3 Body weight gain, food intake, and organ weights in hamsters fed control and the two experimental diets containing 0.5% grape seed proanthocyanidin (0.5% GSP), 1.0% grape seed proanthocyanidin (1.0% GSP).

	Control	0.5% GSP	1.0% GSP
Initial body weight (g)	119.6 ± 6.9	120.5 ± 9.0	120.4 ± 6.3
Final body weight (g)	128.9 ± 13.9	130.4 ± 13.1	132.0 ± 13.7
Food intake (g/day)	11.9 ± 0.4	12.8 ± 0.5	12.9 ± 0.7
Absolute organ weight (g)			
Liver	5.22 ± 0.49	5.14 ± 0.85	5.42 ± 1.05
Kidneys	1.14 ± 0.06	1.12 ± 0.11	1.11 ± 0.11
Heart	0.49 ± 0.05	0.55 ± 0.06	0.51 ± 0.05
Epididymal fat	2.16 ± 0.59	1.81 ± 0.69	2.01 ± 0.40
Perirenal fat	1.12 ± 0.36	1.11 ± 0.45	1.08 ± 0.33
Brain	0.86 ± 0.07	0.95 ± 0.08	0.90 ± 0.09
Relative organ weight (g/100g body weight)			
Liver	4.09 ± 0.63	3.96 ± 0.60	4.10 ± 0.60
Kidneys	0.89 ± 0.13	0.86 ± 0.09	0.84 ± 0.08
Heart	0.39 ± 0.06	0.42 ± 0.05	0.39 ± 0.05
Epididymal fat	1.69 ± 0.53	1.37 ± 0.43	1.51 ± 0.18
Perirenal fat	0.87 ± 0.28	0.84 ± 0.30	0.81 ± 0.18
Brain	0.68 ± 0.08	0.73 ± 0.09	0.69 ± 0.11

Values are expressed as mean ± SD, n=11.

Table 2.4 Changes in serum TC, TG, HDL-C, Non-HDL-C in hamsters fed the control and the two experimental diets containing 0.5% grape seed proanthocyanidin (0.5% GSP), 1.0% grape seed proanthocyanidin (1.0% GSP) for the six weeks.

	Control	0.5% GSP	1.0% GSP
Week 0			
TC (mmol/L)	6.46 ± 0.82	6.41 ± 0.59	6.43 ± 0.65
HDL-C (mmol/L)	2.74 ± 0.19	2.81 ± 0.27	2.69 ± 0.20
Non-HDL-C (mmol/L)	3.72 ± 0.83	3.60 ± 0.49	3.74 ± 0.58
Non-HDL-C/HDL-C	1.37 ± 0.36	1.29 ± 0.18	1.40 ± 0.23
TG (mmol/L)	3.28 ± 1.54	2.44 ± 0.57	3.41 ± 1.81
Week 3			
TC (mmol/L)	6.36 ± 0.80	6.14 ± 0.49	5.62 ± 0.66
HDL-C (mmol/L)	2.57 ± 0.35	2.71 ± 0.30	2.46 ± 0.28
Non-HDL-C (mmol/L)	3.79 ± 0.85	3.40 ± 0.45	3.13 ± 0.70
Non-HDL-C/HDL-C	1.51 ± 0.40	1.26 ± 0.22	1.28 ± 0.34
TG (mmol/L)	4.01 ± 1.59[a]	2.87 ± 0.85[ab]	2.36 ± 0.98[b]
Week 6			
TC (mmol/L)	6.30 ± 0.63[a]	6.05 ± 0.85[ab]	5.39 ± 0.63[b]
HDL-C (mmol/L)	2.66 ± 0.15	2.63 ± 0.40	2.55 ± 0.18
Non-HDL-C (mmol/L)	3.64 ± 0.59[a]	3.45 ± 0.72[ab]	2.85 ± 0.57[b]
Non-HDL-C/HDL-C	1.37 ± 0.23	1.32 ± 0.23	1.12 ± 0.22
TG (mmol/L)	4.62 ± 1.36[a]	4.08 ± 0.98[ab]	3.23 ± 1.10[b]

Data are mean ± SD values.

[a,b]Means in a row with different letters differ significantly, $p<0.05$.

Table 2.5 Fecal excretion of neutral and acidic sterols (mg/hamster/day) in hamsters fed the control and the two experimental diets containing 0.5% grape seed proanthocyanidin (0.5% GSP), and 1.0% grape seed proanthocyandin (1.0% GSP) at week 6.

	Control	**0.5% GSP**	**1.0% GSP**
Neutral sterols (mg/day)			
Coprostanol	1.13 ± 0.75	0.64 ± 0.22	0.46 ± 0.31
Coprostanone	0.05 ± 0.02	0.05 ± 0.02	0.08 ± 0.01
Cholesterol	0.37 ± 0.12	0.36 ± 0.15	1.63 ± 1.25
Dihydrocholesterol	0.27 ± 0.07	0.26 ± 0.07	0.22 ± 0.04
Campersterol	0.07 ± 0.03	0.07 ± 0.01	0.07 ± 0.02
Total	2.08 ± 0.95	1.39 ± 0.33	2.25 ± 1.55
Acidic sterols (mg/day)			
Lithocholic acid	0.46 ± 0.11^{b}	2.23 ± 1.58^{ab}	2.57 ± 1.21^{a}
Deoxycholic acid	0.15 ± 0.08^{b}	0.66 ± 0.41^{a}	0.94 ± 0.53^{a}
Chenodeoxycholic acid + cholic acid	0.26 ± 0.13^{b}	1.11 ± 0.64^{ab}	1.62 ± 0.80^{a}
Ursodeoxycholic acid	0.14 ± 0.04	0.38 ± 0.25	0.54 ± 0.35
Total	1.00 ± 0.32^{b}	4.38 ± 2.86^{ab}	5.67 ± 1.98^{a}

Data are mean ± SD values.

^{a,b}Means in a row with different letters differ significantly, $p<0.05$.

Table 2.6 Cholesterol balance in hamsters fed the control diet, and two experimental diets supplemented with containing 0.5% grape seed proanthocyanidin (0.5% GSP) and 1.0% grape seed proanthocyandin (1.0% GSP) for the six weeks.

	Control	0.5% GSP	1.0% GSP
Week 1			
Cholesterol intake (mg/day)	11.62 ± 0.37	12.33 ± 0.69	12.07 ± 0.73
total neutral sterol (mg/day)	1.70 ± 0.42	1.42 ± 0.43	1.63 ± 0.36
total acidic sterol (mg/day)	2.08 ± 0.70	2.98 ± 1.14	3.13 ± 1.45
cholesterol retained (mg/day)	7.84 ± 0.47	7.93 ± 1.82	7.31 ± 1.98
Cholesterol retain/cholesterol intake (%)	67.60 ± 4.94	63.85 ± 11.73	60.27 ± 14.70
Week 3			
Cholesterol intake (mg/day)	12.26 ± 1.02	12.62 ± 0.83	12.38 ± 0.72
total neutral sterol (mg/day)	1.56 ± 0.72	1.41 ± 0.38	1.99 ± 0.93
total acidic sterol (mg/day)	1.67 ± 0.86^{b}	4.03 ± 2.69^{ab}	4.53 ± 0.92^{a}
cholesterol retained (mg/day)	9.04 ± 1.51^{b}	7.19 ± 2.91^{ab}	5.86 ± 1.11^{a}
Cholesterol retain/cholesterol intake (%)	73.65 ± 9.51^{a}	57.12 ± 22.49^{ab}	47.16 ± 7.46^{b}
Week 6			
Cholesterol intake (mg/day)	11.21 ± 0.69	12.35 ± 0.54	12.79 ± 0.49
total neutral sterol (mg/day)	2.08 ± 0.95	1.39 ± 0.33	2.25 ± 1.55
total acidic sterol (mg/day)	1.00 ± 0.32^{b}	4.38 ± 2.86^{ab}	5.67 ± 1.98^{a}
cholesterol retained (mg/day)	8.13 ± 0.73	6.59 ± 2.52	4.87 ± 2.86
Cholesterol retain/cholesterol intake (%)	72.80 ± 8.67^{a}	53.68 ± 20.62^{ab}	38.22 ± 22.14^{b}

Data are mean ± SD values.

^{a,b}Means in a row with different letters differ significantly, $p<0.05$.

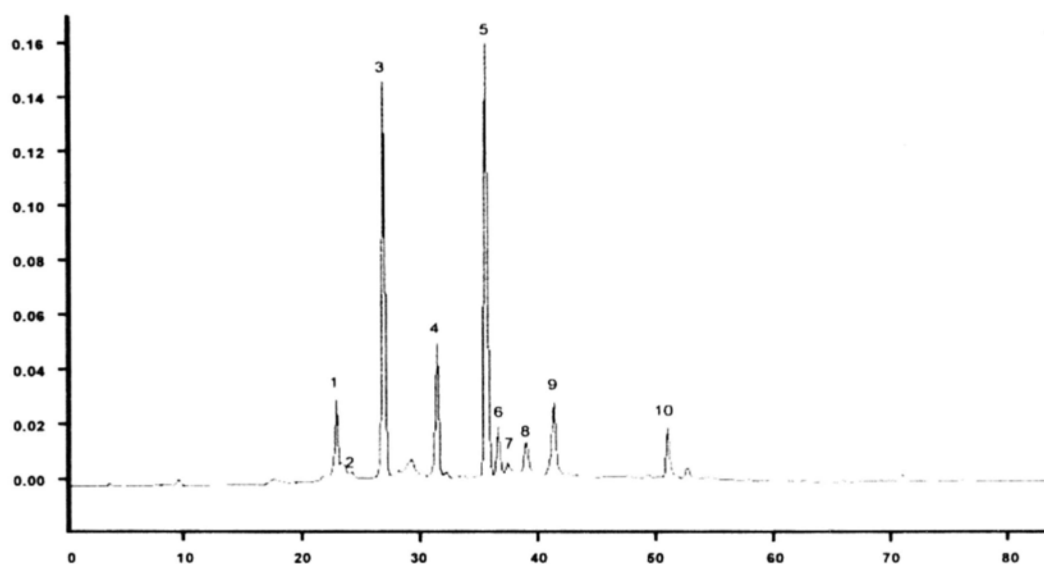


Figure 2.1

HPLC chromatogram of GSP. Identification of peaks: peaks 1 and 2, dimmer procyanidins; peak 3, catechin; peak 4, procyanidin B2; peak 5, epicatechin; peaks 6 and 9; dimmers esterified gallic acid; peaks 7 and 8, trimers esterfied with gallic acid; and peak 10, trimers esterified with gallic acid.

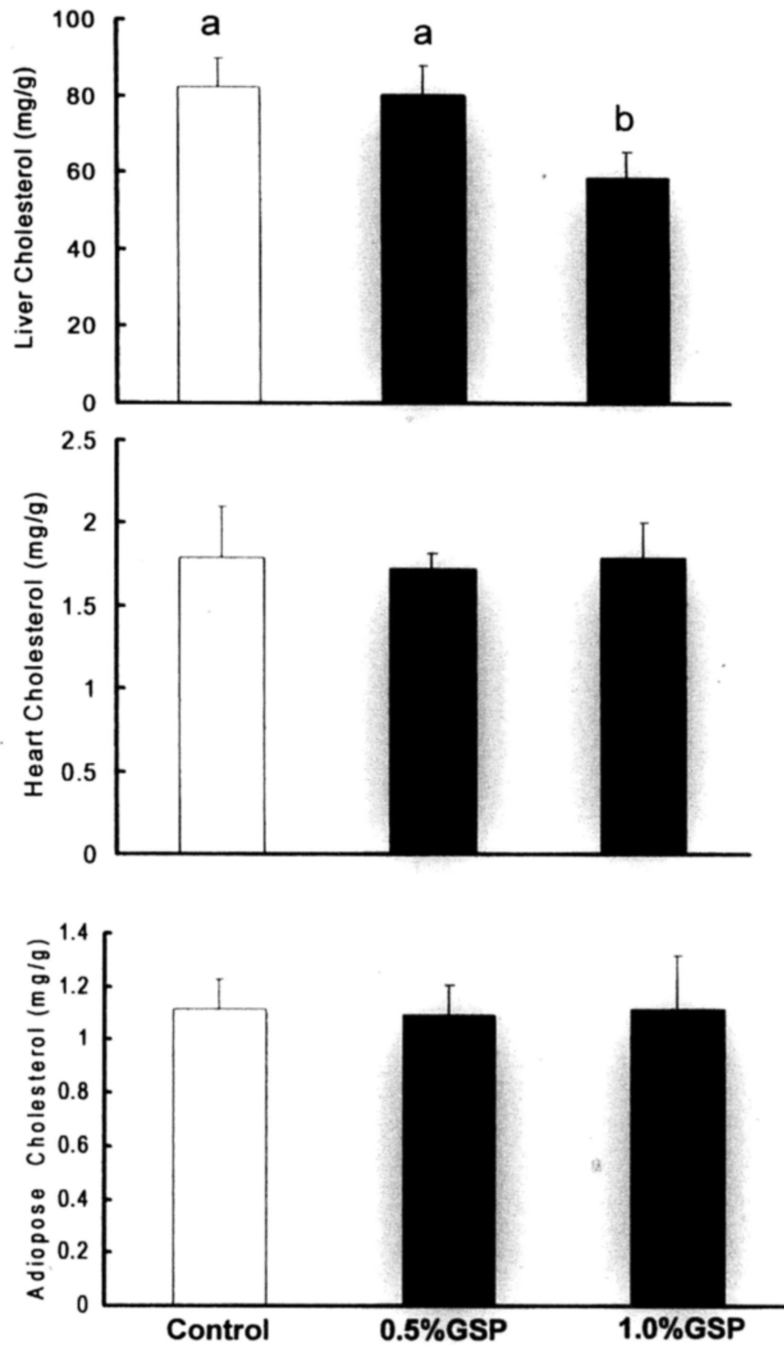


Figure 2.2

Effect of GSP on cholesterol content in the liver, heart and adipose tissue. [a,b]Means with different letters differ significantly, $p<0.05$.

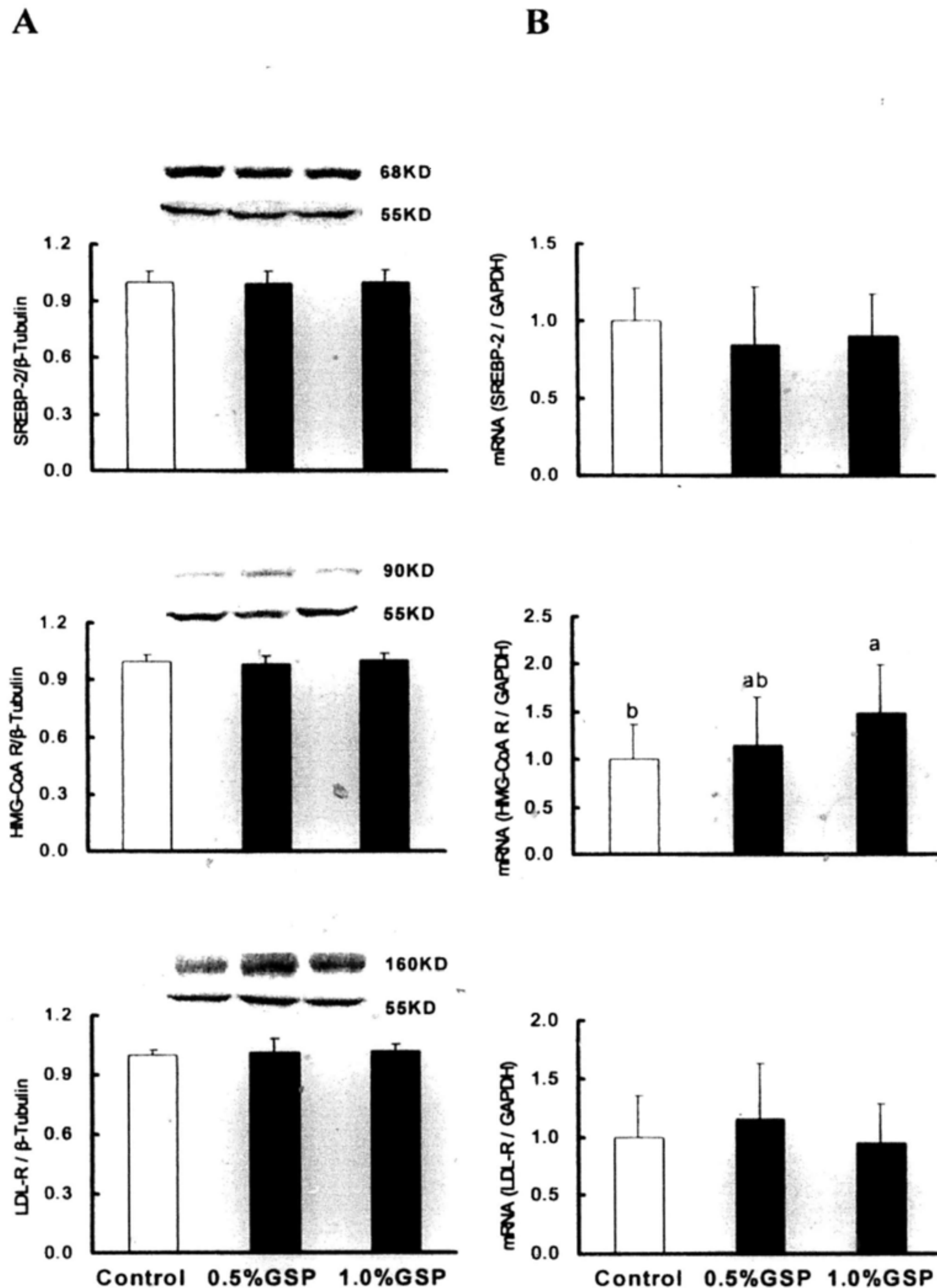
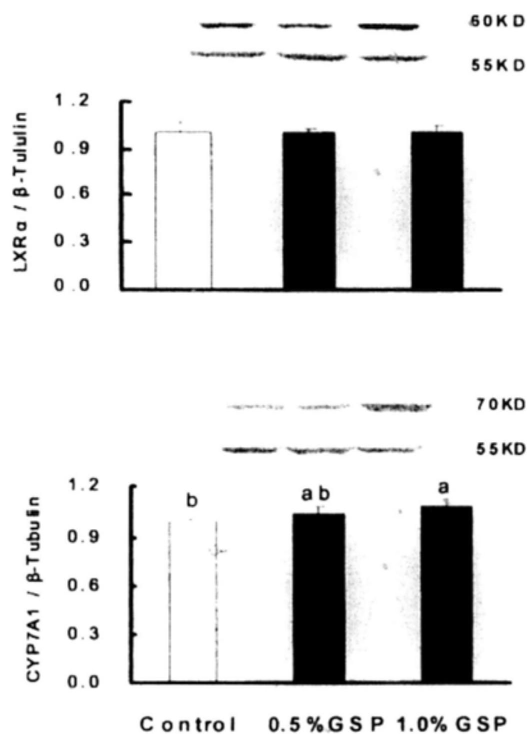


Figure 2.3

The relative immunoreactive mass (A) and mRNA (B) of hepatic SREBP-2, HMG-CoA-R, LDLR in hamsters fed the control diet, and two experimental diets supplemented with containing 0.5% grape seed proanthocyanidin (0.5% GSP) and 1.0% grape seed proanthocyandin (1.0% GSP) for the six weeks. Values are expressed as means ± SD (n=11) with those for control group being arbitrarily taken as one. [a,b]Means with different superscrip letters differ significantly, $p<0.05$.

A



B

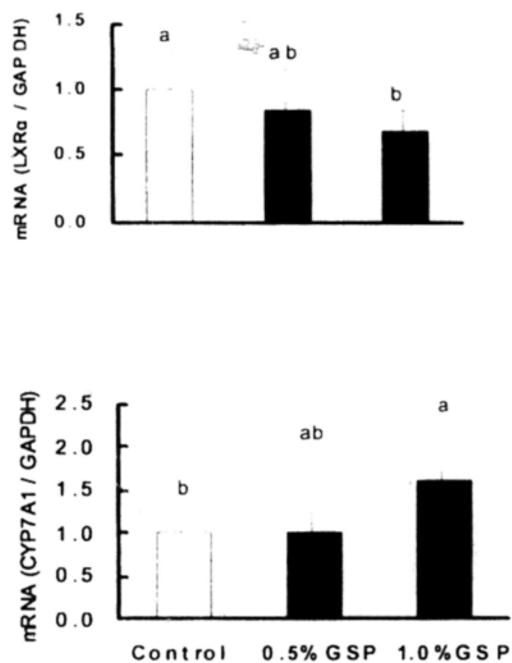


Figure 2.4

The relative immunoreactive mass (A) and mRNA (B) of hepatic LXRα and CYP7A1 in hamsters fed the control diet, and two experimental diets supplemented with containing 0.5% grape seed proanthocyanidin (0.5% GSP) and 1.0% grape seed proanthocyandin (1.0% GSP) for the six weeks. Values are expressed as means ± SD (n=11) with those for control group being arbitrarily taken as one. [a,b]Means with different superscrip letters differ significantly, $p<0.05$.

2.5 Discussion

The present study was to investigate the hypocholesterolemic activity of GSP using hamsters as an animal model. In this regard, supplementation of GSP into diet reduced not only plasma TC and non-HDL-C levels but also plasma TG in a dose-dependent manner (Table 2.4). The present study clearly demonstrated that supplementation of 1.0% GSP for six weeks could decrease serum TC by 15% and TG by 30% in hamsters. Results are in agreement with those reported in rabbits (Frederiksen et al, 2007), hamsters (Vinson et al., 2002; Auger et al., 2004) and rats (del Bas et al., 2005). However, little is known about the mechanism by which GSP decreases blood cholesterol level. The present study was the first of its kind to characterize interaction of dietary GSP with SREBP-2, HMG-CoA-R, LDLR, LXRα and CYP7A1 in hamsters fed a high-cholesterol diet.

Cholesterol is mainly eliminated from the body via conversion of cholesterol to bile acids. The present study clearly demonstrated that dietary GSP was able to increase the excretion of bile acids by 3-4 folds (Table 2.5). This was partially mediated by up-regulation of CYP7A1 in both transcriptional and translational levels (Figure 2.4). The present study further demonstrated that GSP had no effect on the protein level of LXRα but it decreased its mRNA level. The observation is in agreement with that of del Bas et al. (del Bas et al., 2005), who only studied the effect of GSP on the mRNA levels of these proteins and enzymes involved in cholesterol metabolism in rats, finding that GSP was able to up-regulate CYP7A1 with a slight down-regulation or no change in LXRα. In general, LXRα is effective to up-regulate CYP7A1 transcription in rats but it has no or much less effect on CYP7A1 in hamsters (Chiang et al., 2001). The present study in hamsters together with the report in rats (del Bas et al., 2005) found the change pattern in expression of LXRα was contrary to that of CYP7A1. This discrepancy could be explained by a

decreased level of liver cholesterol associated with supplementation of GSP in diet (Figure 2.2), as LXRα is a cholesterol sensor and its gene expression was partially down-regulated in response to a decrease in hepatic cholesterol concentration (Thornton et al., 2008). It should be pointed out that the CYP7A1 transcription is regulated by not only LXRα but also other factors including farnesoid X receptor (FXR), a repressor of CYP7A1 gene expression (del Bas et al., 2005). Therefore, dietary GSP could up-regulate the expression of CYP7A1 without necessarily up-regulating LXRα in hamsters.

The present study investigated the effect of dietary GSP on SREBP-2, LDLR and HMG-CoA-R, finding that GSP had no effect on their protein and mRNA levels except it increased only the mRNA HMG-CoA-R. This is in agreement with the results of del Bas et al. (del Bas et al., 2005), who demonstrated that GSP could up-regulate the mRNA HMG-CoA-R by > 40%. The observed increase in the mRNA HMG-CoA-R could be explained by the decrease in cholesterol concentration in the liver of the 1.0GSP group. The HMG-CoA-R is sensitive to the cholesterol concentration in the liver and become up-regulated if the liver cholesterol is reduced. In addition, hamsters have a diurnal pattern in expression of SREBP-2, LDLR and HMG-CoA-R with their activity in midnight being several times higher than that in midday (Greenough et al., 1982). Perhaps, the abundance in HMG-CoA-R, SREBP-2, and LDLR was already very low because hamsters in the present study were sacrificed between 9:00-11:00am, so that cholesterol catabolism rate was nil and no effect of dietary GSP in these proteins could be seen in hamsters after the overnight fasting.

The present study is the first of its kind to study the effect of GSP on cholesterol balance. GSP could decrease the cholesterol absorption. Total intake of cholesterol was compared with its excretion with neutral and acidic sterols (Table 2.6). When

net cholesterol equivalent retained was calculated by difference between intake and excretion of both neutral and acidic sterols, it was found that net cholesterol retention was the most in the control followed by 0.5% GSP, and 1.0% GSP groups in a decreasing order. The apparent cholesterol absorption was calculated in an equation [(cholesterol intake - excretion of neutral and acidic sterols)/cholesterol intake]. It appeared that GSP decreased the cholesterol absorption in a dose-dependent manner.

In summary, the present study systematically investigated the effect of dietary GSP on SREBP-2, LDLR, HMG-CoA-R, LXR and CYP7A1 at both transcriptional and translational levels in hamsters. It was found that GSP at 1.0% level markedly increased the fecal excretion of bile acids by 2-3 folds mediated by up-regulation of CYP7A1. In addition, dietary 1.0% GSP reduced significantly the apparent cholesterol absorption from 72.80% to 38.22%. The data suggest that enhancement of bile acid excretion is the major mechanism by which GSP decreases plasma cholesterol.

Chapter 3

Frequent Cholesterol Intake is Associated with Elevation of Plasma Total Cholesterol Level

3.1 Introduction

Cholesterol has been long blamed as a culprit in the incidence of CHD. Epidemiological studies have demonstrated that elevated levels of plasma TC and LDL-C are the major risk factors, while high concentrations of plasma HDL-C and a low ratio of TC to HDL-C are protective ones against CHD. It is known that eating frequency influences blood lipoprotein profile and glucose metabolism (Titan et al., 2001). Plasma lipids including cholesterol itself have been shown to increase when animals are switched from a nibbling diet to a gorging diet pattern (Titan et al., 2001; Juhel et al., 2000). In humans, regular meal frequency has beneficial effects on fasting lipid and postprandial insulin profile (Farshchi et al., 2005; Jenkins et al., 1989; Arnold et al., 1993). However, results from other studies have been inconclusive (Murphy et al., 1996; Rashidi et al., 2003).

In a typical Western diet consumed by the average adult, nearly 1,200-1,700 mg cholesterol enters the small intestinal lumen each day. And 300-500 mg of this cholesterol comes from the diet and the remainder is mainly derived from bile each day (Grundy et al., 1983; Grundy et al., 1972). Then, about half of the cholesterol is excreted in feces, the remainder is absorbed into the intestinal cells, then esterified and packaged into chylomicrons. As CMs leave the intestine, their cholesterol is transported through the lymphatic system to the blood and the liver. Liver is the main site for cholesterol de novo synthesis, also it's the target organ for degradation and excretion of cholesterol to biliary cholesterol and bile acids to digest lipids, and

most bile acid could be reabsorped through hepatic portal vessels, and only a little bile acid could be excreted by feces. In addition, the liver can receive excess cholesterol from blood and other tissues through the reverse cholesterol transport system (Figure 3.1). The underlying metabolism has been described in section 1.4.

3.2 Objective

Despite extensive investigation on the link between dietary cholesterol and plasma lipoprotein profile, little is known of how cholesterol intake frequency interacts with the 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, LDLR and CYP7A1 in hamsters.

3.3 Materials and methods

3.3.1 Diets

All diets were prepared as previously described (Lam et al., 2008). A cholesterol-free diet was prepared by mixing the following ingredients (g/kg 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 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 −20 °C.

3.3.2 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. 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) and housed in wire-bottomed cages at 23°C in an animal room with 12-h light–dark cycle. All the hamsters were adapted to gavage-feeding 3 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, one group was maintained on a 0.1% cholesterol diet (CD) throughout the entire study (high cholesterol intake frequency). It was found that CD hamsters consumed about 10 g of foods daily which contained 9 mg of cholesterol. The other two groups were fed a cholesterol free diet with a gavage-administration of 9 mg of cholesterol per day. For the regular cholesterol intake frequency group (C-3), hamsters were daily gavage-administered 3 mg

cholesterol in 0.5 ml of 3% starch solution for three times at 9:00 am, 4:00 pm, 10:00 pm, respectively, when were the three peak times of food consumption by hamsters. For the low cholesterol intake frequency group (C-1), hamsters were daily gavage-administered 9 mg cholesterol in 0.5 ml of 3% starch solution for one time; for the other two times, C-1 group was orally given 0.5 ml of 3% starch solution containing no cholesterol. Similarly, CD group was also gavage-administered three times 0.5 ml of 3% starch solution containing no cholesterol. 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 week 2, 4 and 6 following food deprivation for 14 h and light anaesthesia, using a mixture of ketamine, xylazine and saline (v/v/v, 4:1:5). The blood was centrifuged at 1,000 × 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 the hamsters were kept for 3 days to allow for recovery and then euthanized by carbon dioxide suffocation after 14 h food deprivation. Liver, heart, kidney, adipose tissues (perirenal and epididymal pads) were removed, washed in saline, and weighed. The first 5 cm of duodenum was discarded, and the following 30 cm of the small intestine was kept with the first 15cm 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.

3.3.3 Determination of plasma lipoproteins

The analyses were similarly conducted as previously described in section 2.3.4, Chapter 2.

3.3.4 Measurement of organ cholesterol and fecal neutral and acidic sterols

Materials and experimental procedures were described in section 2.3.5 and 2.3.6, Chapter 2.

3.3.5 Western blotting analysis of liver SREBP, LDLR, HMG-CoA-R, LXRα and CYP7A1

The analysis were similarly conducted as previously described in section 2.3.7, chapter 2 except that data on abundance of SREBP-2, LDLR, HMG-CoA-R, LXRα and CYP7A1 were normalized with β-actin (Santa Cruz Biotechnology, Inc., California, USA) not β-tubulin.

3.3.6 Real-time PCR analysis of mRNA for liver SREBP-2, LDLR, HMG-CoA R, LXR, CYP7A1 and small intestine NPC1L1, ABCG5, ABCG8, ACAT2, MTP

Total mRNA levels in the liver SREBP-2, LDLR, HMG-CoA R, LXR and CYP7A1 were quantified as previously described (Lam et al., 2008). In brief, total RNA was extracted and converted to complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription kit (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. 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 used for real-time PCR for the liver GAPDH, CYP7A1,

HMG-CoA-R, LDLR, SREBP and LXRα (Table 2.2, Chapter 2) in hamsters, whereas for the small intestinal NPC1L1, ABCG5, ABCG8, ACAT2, MTP and 18S, SYBR green was used as a fluorophore (Table 3.1). The reaction mixture was subject to thermal cycling under the following conditions: heating up to 95°C in 20 seconds, followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds. 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).

3.3.7 Measurement of intestinal ACAT activity and Western blotting analysis of intestinal MTP

ACAT activity was quantified as we previously described (Yeung et al, 2000). 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, 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 cholesteryl ester were separated on a thin-layer silica gel plate (Merck, NJ, USA) 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 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.

Total intestinal microsome proteins were separated on a 7% SDS-PAGE gel and transferred to PVDF membranes (Millipore, Billerica, MA, USA) using a

semi-dry transfer system. Membranes were then blocked in 5% nonfat milk Tris-buffered saline with Tween-20 for 1 hour and overnight at 4°C in the same solution containing anti–MTP polyclonal antibody (BD Biosciences, Palo Alto, CA, USA). The membrane was then incubated for one hour at 4°C in diluted horseradish peroxidase-linked Goat-anti-mouse IgG (Santa Cruz Biotechnology, Inc. California, USA). Then, membranes were developed with ECL enhanced chemiluminescence agent (Santa Cruz Biotechnology, Inc., California, USA) and subjected to autoradiography on SuperRX medical X-ray film (Fuji, Tokyo, Japan). Densitometry was quantified using the BioRad Quantity one® software (BioRad Laboratories, Hercules, USA). Data on abundance of MTP were normalized with β-actin (Santa Cruz Biotechnology, Inc., California, USA).

3.3.8 Statistics

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

3.4 Results

3.4.1 Effect of cholesterol consumption frequency on food intake, body and organ weights

No significant differences in the final body weight were seen among the three groups. Similarly, there were no significant differences in food intakes among the three groups. The ratio of liver, heart, epididymal fat and perirenal fat to the final body weight was similar among the three groups.

3.4.2 Effect of cholesterol consumption frequency on 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 3.2). At the end of week 2, plasma TG demonstrated a decreasing trend among the CD, C-3 and C-1 groups (Table 3.2). 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 CD, C-3 and C-1 groups, respectively.

3.4.3 Effect of cholesterol consumption frequency on organ cholesterol

No differences in heart cholesterol content were seen among the three groups. In contrast, cholesterol content in the liver, kidney and adipose tissues demonstrated a decreasing trend in the CD, C-3 and C-1 groups (Figure 3.2).

3.4.4 Effect of cholesterol consumption frequency on cholesterol balance and

excretion of fecal neutral and acidic sterols

Concentration of individual neutral and acidic sterols in the feces of the hamsters at week 2, 4, 6 is shown in Table 3.3. In general, C-3 and C-1 groups had significantly lower fecal coprostanone and higher cholesterol output than CD group, and at week 6, total fecal neutral sterols of C-3 and C-1 groups was 3.85 and 3.50 fold higher than that of CD group. The statistical analysis did not find any difference in fecal output of individual acidic and total acidic sterols.

Total intake of cholesterol was compared with its excretion in neutral and acidic sterols (Table 3.4). 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 CD group was greatest followed by that in C-3 and C-1 groups. The apparent cholesterol absorption was calculated by an 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.

3.4.5 Effect of cholesterol consumption frequency on immunoblot and mRNA analyses of liver SREBP, LDLR, 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 3.3). Although the immunoblot analysis showed that frequency of cholesterol intake had no effect on the protein mass of LDLR, real time PCR analysis demonstrated that increasing cholesterol intake frequency could down-regulate the expression of mRNA LDLR (Figure 3.3).

3.4.6 Effect of cholesterol consumption frequency on 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 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 C-3 and C-1 groups (Figure 3.4).

3.4.7 Effect of cholesterol consumption frequency on 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 3.5). C-1 group had its intestinal ACAT activity reduced by 58.4% and 52.8%, respectively, compared with CD and C-3 groups. The immunoblot analysis indicated that the protein mass of intestinal MTP of C-1 group was significantly lower than that of both CD and C-3 group.

Table 3.1 Quantitative real-time PCR primers used to measure the intestinal RNA levels of NPC1L1, ABCG5/8, ACAT2 and MTP

Gene	NCBI accession number or references	Forward primer 5'→ 3'	Reverse primer 5' → 3'
SYRB Green			
NPC1L1	DQ897680	CCTGACCTTTATAGAACTCACCACAGA	GGGCCAAAATGCTCGTCAT
ABCG5	(Field et al., 2004)	TGATTGGCAGCTATAATTTTGGG	GTTGGGCTGCGATGGAAA
ABCG8	(Field et al., 2004)	TGCTGGCCATCATAGGGAG	TCCTGATTTCATCTTGCCACC
ACAT2	(Valasek et al., 2008)	CCGAGATGCTTCGATTTGGA	GTGCGGTAGTAGTTGGAGAAGGA
MTP	(Qin et al., 2004)	GTCAGGAAGCTGTGTCAGAATG	CTCCTTTTTCTCTGGCTTTTCA
18S	M33069	TAAGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC
Cyclophilin	(Valasek et al., 2008)	CAAATGCTGGACCAAACACA	CAGTCTTGGCGGTGCAGAT

Table 3.2 Changes in plasma TC, TG, HDL-C, Non-HDL-C in hamsters fed the 0.1% cholesterol diet (CD), the cholesterol-free diet with gavage-administration of 3 mg cholesterol for 3 times per day (C-3) or the cholesterol-free diet with gavage-administration of 9 mg cholesterol one time per day (C-1).

	CD	C-3	C-1	*P*
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

Data are expressed as mean ± SD, n=11. [a,b]Means in a row with different letters differ significantly, $p < 0.05$.

Table 3.3 Change in fecal output of individual neutral and acidic sterols in hamsters fed the 0.1% cholesterol diet (CD), no cholesterol diet with gavage-fed 3mg cholesterol 3 times per day (C-3) and no cholesterol diet with gavage-fed 9mg cholesterol 1time per day (C-1)[1], respectively

	CD	C-3	C-1	*P*
Week 2				
Neutral sterols (mg/day)				
Coprostanol	0.46 ± 0.11	0.82 ± 0.55	0.89 ± 0.58	0.33
Coprostanone	0.04 ± 0.01[ab]	0.03 ± 0.01[b]	0.04 ± 0.01[a]	0.10
Cholesterol	0.41 ± 0.07[c]	2.95 ± 0.39[b]	3.72 ± 0.53[a]	<0.01
Dihydrocholesterol	0.20 ± 0.04	0.23 ± 0.04	0.26 ± 0.05	0.23
Campersterol	0.06 ± 0.01[b]	0.07 ± 0.02[ab]	0.08 ± 0.01[a]	0.45
Total	1.17 ± 0.20[b]	4.09 ± 0.69[ab]	4.99 ± 0.49[a]	<0.01
Acidic sterols (mg/day)				
Lithocholic acid	0.85 ± 0.13	1.12 ± 0.41	1.03 ± 0.28	0.38
Deoxycholic acid	0.16 ± 0.04	0.17 ± 0.09	0.16 ± 0.07	0.97
Chenodeoxycholic acid	0.31 ± 0.06	0.37 ± 0.22	0.25 ± 0.09	0.51
Cholic acid	0.27 ± 0.10	0.50 ± 0.54	0.29 ± 0.16	0.40
ursodeoxycholic acid	0.20 ± 0.06	0.23 ± 0.07	0.19 ± 0.04	0.68
Total	1.79 ± 0.39	2.38 ± 1.31	1.92 ± 0.51	0.53
Week4				
Neutral sterols (mg/day)				
Coprostanol	0.37 ± 0.03	0.52 ± 0.28	0.80 ± 0.47	0.15
Coprostanone	0.03 ± 0.01	0.02 ± 0.01	0.10 ± 0.11	0.19
Cholesterol	0.44 ± 0.10[b]	4.46 ± 0.95[a]	3.71 ± 0.72[a]	<0.01
Dihydrocholesterol	0.21 ± 0.03	0.26 ± 0.08	0.28 ± 0.08	0.28
Campersterol	0.05 ± 0.01[b]	0.07 ± 0.02[a]	0.06 ± 0.01[a]	0.02
Total	1.10 ± 0.15[b]	5.34 ± 1.29[a]	4.95 ± 1.22[a]	<0.01
Acidic sterols (mg/day)				
Lithocholic acid	0.60 ± 0.14	0.61 ± 0.19	0.72 ± 0.22	0.58
Deoxycholic acid	0.13 ± 0.03	0.14 ± 0.03	0.18 ± 0.07	0.30
Chenodeoxycholic acid	0.14 ± 0.05	0.22 ± 0.05	0.16 ± 0.12	0.48
Cholic acid	0.28 ± 0.22	0.14 ± 0.06	0.42 ± 0.35	0.96
ursodeoxycholic acid	0.14 ± 0.05	0.13 ± 0.06	0.11 ± 0.04	0.79
Total	1.29 ± 0.35	1.23 ± 0.33	1.59 ± 0.62	0.48

Week 6	**CD**	**C-3**	**C-1**	***P***
Neutral sterols (mg/day)				
Coprostanol	0.46 ± 0.17	0.50 ± 0.08	0.67 ± 0.56	0.63
Coprostanone	0.04 ± 0.02[a]	0.02 ± 0.01[b]	0.02 ± 0.01[b]	0.08
Cholesterol	0.51 ± 0.09[b]	3.44 ± 0.79[a]	3.97 ± 0.28[a]	<0.01
Dihydrocholesterol	0.23 ± 0.05	0.23 ± 0.04	0.26 ± 0.06	0.73
Campersterol	0.05 ± 0.02	0.05 ± 0.01	0.06 ± 0.02	0.44
Total	1.29 ± 0.32[b]	4.23 ± 0.89[a]	4.98 ± 0.66[a]	<0.01
Acidic sterols (mg/day)				
Lithocholic acid	0.73 ± 0.21	0.70 ± 0.27	0.77 ± 0.11	0.91
Deoxycholic acid	0.23 ± 0.08	0.20 ± 0.10	0.19 ± 0.01	0.80
Chenodeoxycholic acid	0.25 ± 0.06	0.23 ± 0.10	0.21 ± 0.12	0.70
Cholic acid	0.25 ± 0.11	0.20 ± 0.14	0.19 ± 0.07	0.80
ursodeoxycholic acid	0.17 ± 0.05	0.15 ± 0.02	0.18 ± 0.03	0.43
Total	1.63 ± 0.48	1.48 ± 0.55	1.54 ± 0.24	0.89

[1]Values are means ± SD, n=10. [a,b,c] Means in a row for a given week with different superscript letters differ significantly, $p < 0.05$.

Table 3.4 Changes in daily total cholesterol intake (mg), fecal excretion of total neutral sterols (mg), total acidic sterols (mg), and cholesterol balance in hamsters fed the 0.1% cholesterol diet(CD), no cholesterol diet with gavage-fed 3mg cholesterol 3times per day (C-3) and no cholesterol diet with gavage-fed 9mg cholesterol 1time per day (C-1)[1], respectively

	CD	C-3	C-1	*P*
Week 2				
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 intake (mg)	8.54 ± 0.45^b	9.00 ± 0.00^a	9.00 ± 0.00^a	0.36
Cholesterol retained (mg)	5.58 ± 0.56^a	2.53 ± 1.23^b	2.09 ± 0.86^b	<0.01
Cholesterol retain/cholesterol intake (%)	65.18 ± 3.64^a	28.07 ± 13.65^b	23.23 ± 9.60^b	<0.01
Week 4				
Neutral sterol (mg)	1.10 ± 0.15^b	5.34 ± 1.29^a	4.95 ± 1.22^a	<0.01
Acidic sterol (mg)	1.29 ± 0.35	1.23 ± 0.33	1.59 ± 0.62	0.48
Cholesterol intake (mg)	9.00 ± 0.71	9.00 ± 0.00	9.00 ± 0.00	1.00
Cholesterol retained (mg)	6.62 ± 0.74^a	2.43 ± 1.47^b	2.46 ± 1.70^b	<0.01
Cholesterol retain/cholesterol intake (%)	73.39 ± 4.01^a	26.99 ± 16.32^b	27.34 ± 18.85^b	<0.01
Week 6				
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 intake (mg)	9.64 ± 0.44^a	9.00 ± 0.00^b	9.00 ± 0.00^b	<0.01
Cholesterol retained (mg)	6.72 ± 0.65^a	3.29 ± 1.23^b	2.48 ± 0.68^b	<0.01
Cholesterol retain/cholesterol intake (%)	69.83 ± 7.14^a	36.51 ± 13.64^b	27.59 ± 7.58^b	<0.01

[1]Values are means ± SD, n=10. ^{a,b,c} Means in a row for a given week with different superscript letters differ significantly, $p < 0.05$.

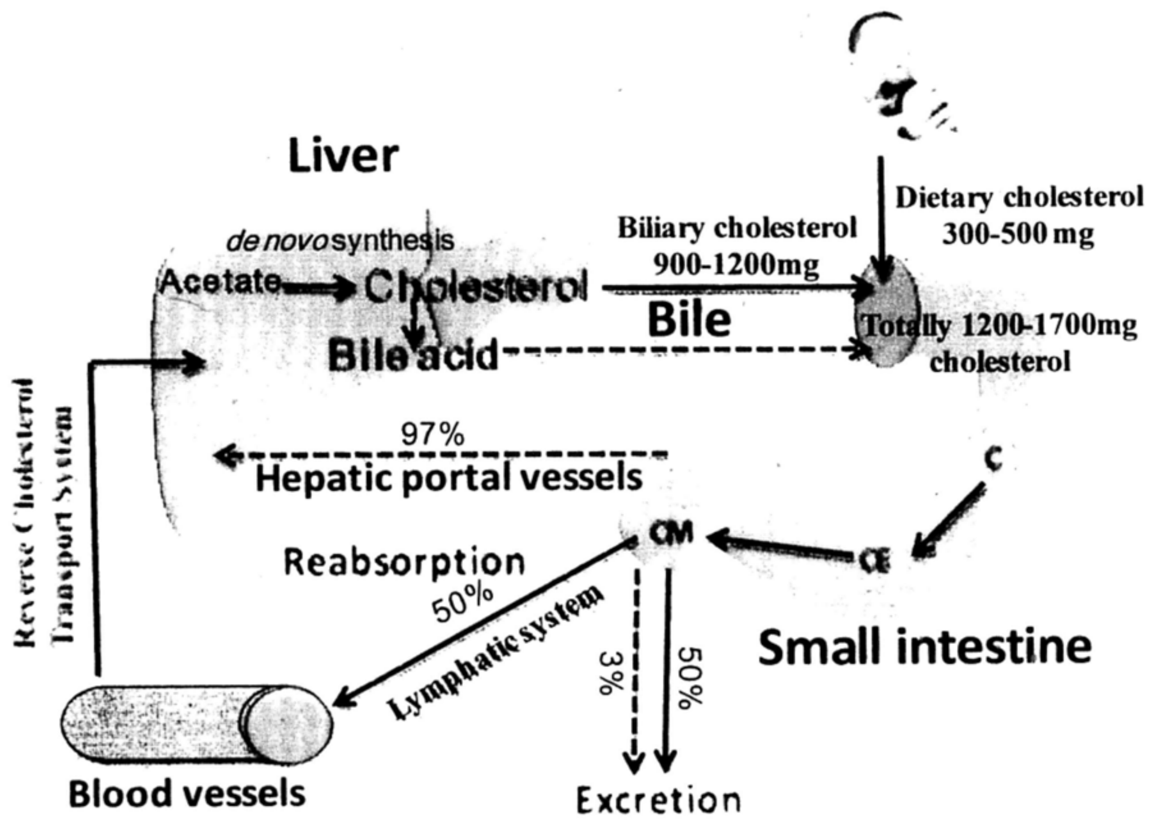


Figure 3.1

Cholesterol homeostasis in human body.

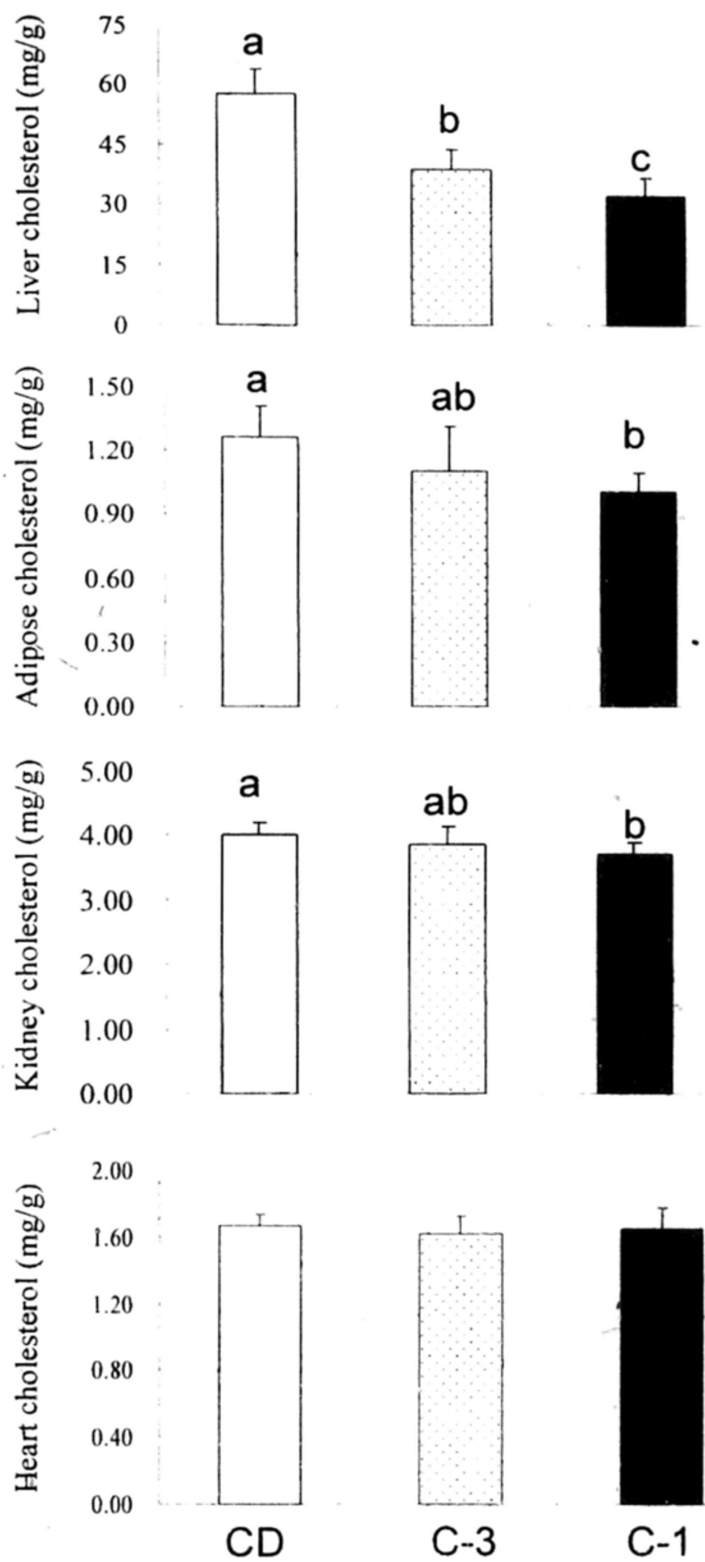


Figure 3.2

Effect of frequent cholesterol intake on cholesterol content in the liver, adipose tissue, kidney and heart. [a,b]Means with different letters differ significantly, $p<0.05$

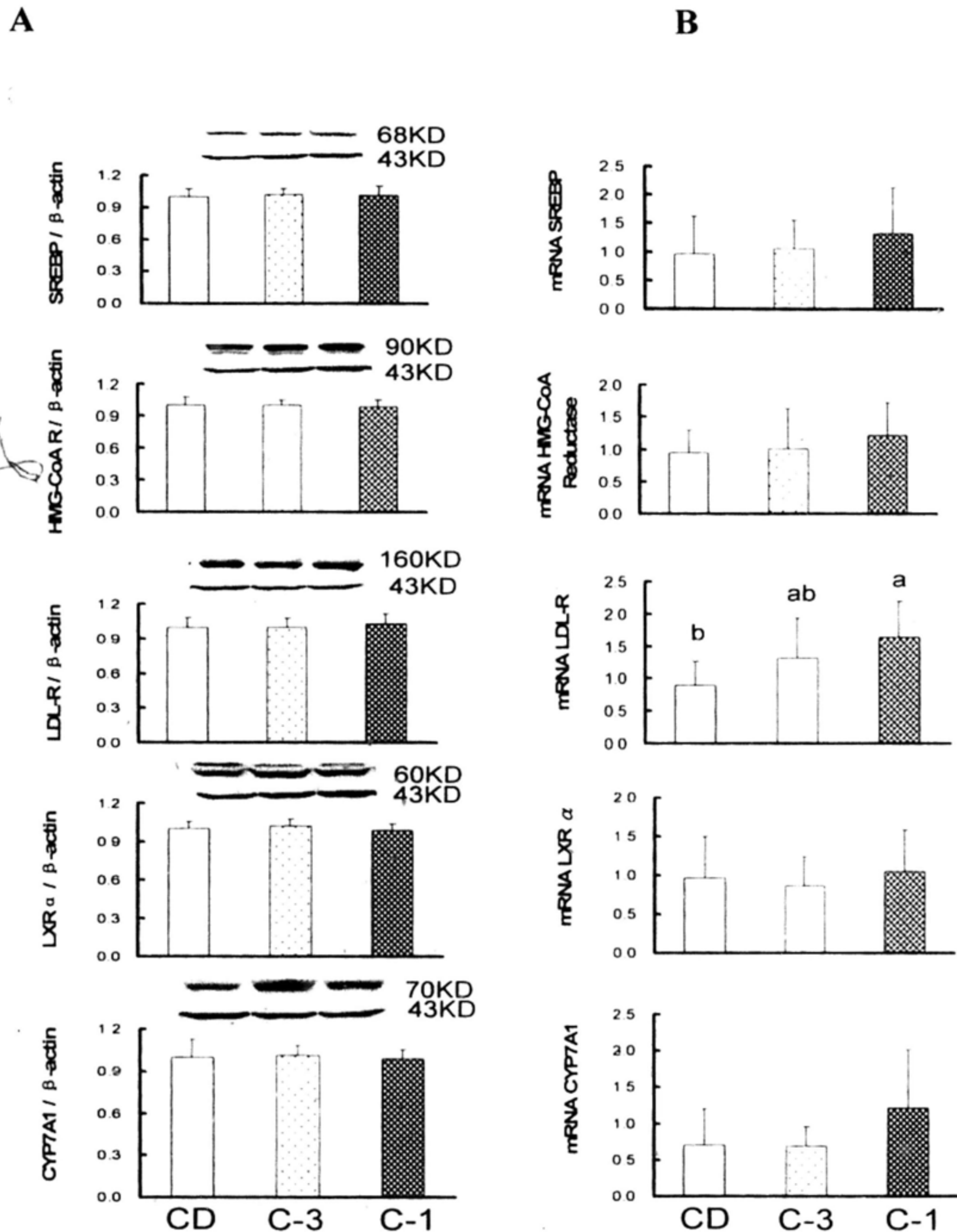


Figure 3.3

The relative immunoreactive mass (A) and mRNA (B) of hepatic SREBP-2, HMG-CoA-R, LDLR, LXRα and CYP7A1 in hamsters fed the 0.1% cholesterol diet (CD), the cholesterol-free diet with gavage-administration of 3 mg cholesterol for 3 times per day (C-3) or the cholesterol-free diet with gavage-administration of 9 mg cholesterol one time per day (C-1). Values are expressed as means±SD (n=11) with those for CD group being arbitrarily taken as one. [a,b]Means with different superscrip letter differ sighnificantly, $p<0.05$.

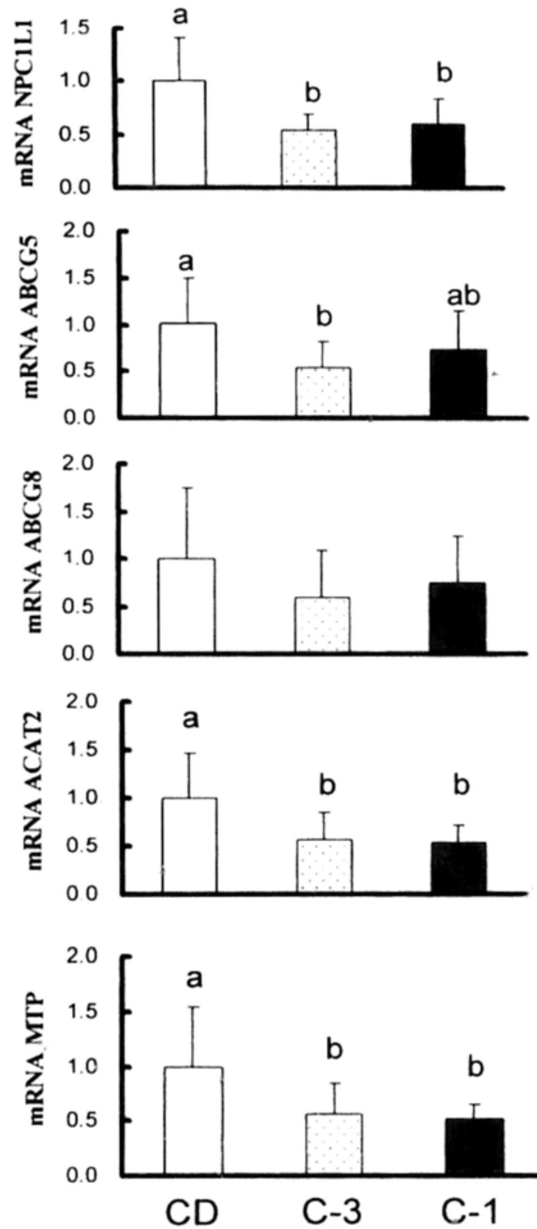


Figure 3.4

The relative mRNA NPC1L1, ABCG5/8, ACAT2 and MTP in hamsters fed the 0.1% cholesterol diet (CD), the cholesterol-free diet with gavage-administration of 3 mg cholesterol for 3 times per day (C-3) or the cholesterol-free diet with gavage-administration of 9 mg cholesterol one time per day (C-1). Values are expressed as means±SD (n=11) with those for CD group being arbitrarily taken as one. [a,b]Means with different superscrip letter differ sighnificantly, $p<0.05$.

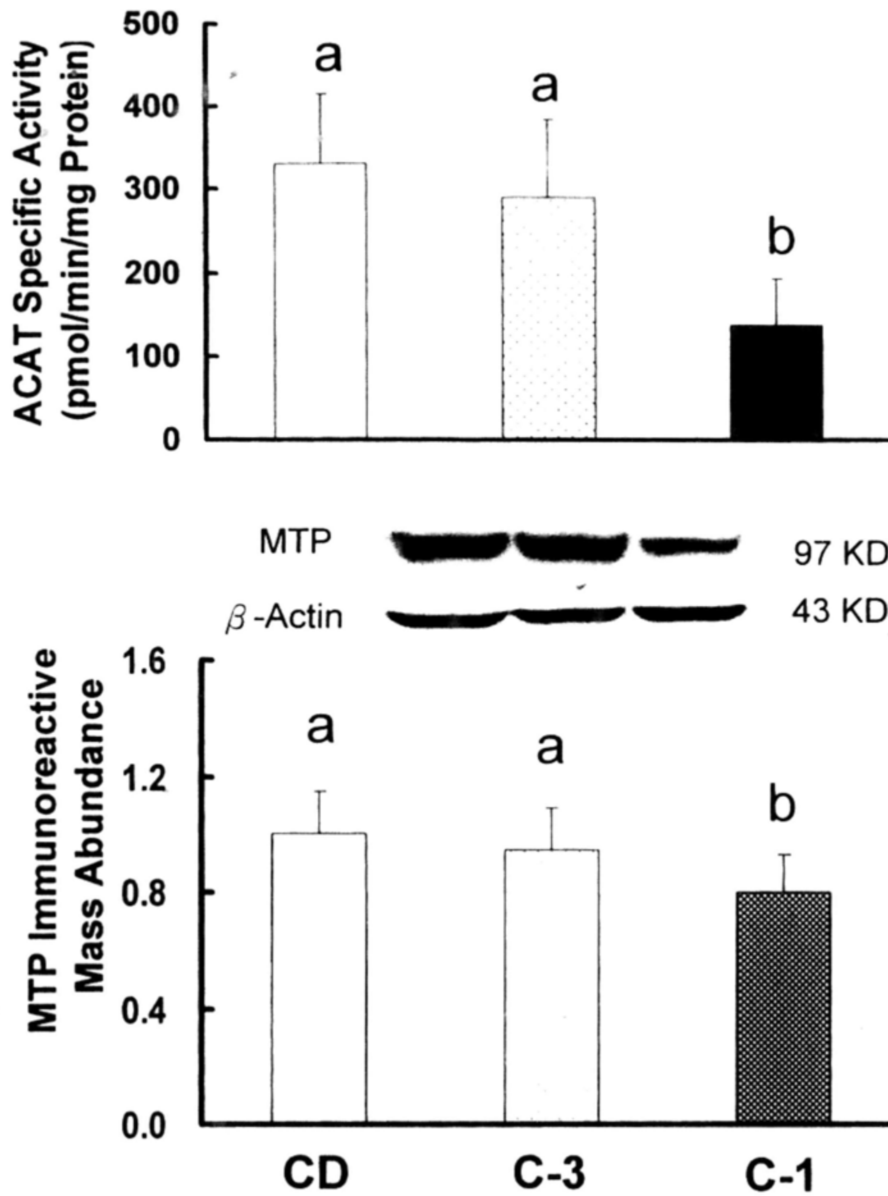


Figure 3.5

Intestinal 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 cholesterol for 3 times per day (C-3) or the cholesterol-free diet with gavage-administration of 9 mg cholesterol one time per day (C-1). [a,b]Means with different letters differ significantly, $p<0.05$.

3.5 Discussion

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, whereas other people taking cholesterol more or less similar among the three meals. 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. In the present study, hamsters were given daily 9 mg of cholesterol either in diet (CD) or 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, TC/HDL-C ratio and TG in association with the cholesterol intake frequency (Table 3.2). It is the first time to demonstrate that the increasing cholesterol intake frequency increased the apparent cholesterol absorption (Table 3.4). This effect was reflected on the observation that C-1 group excreted most fecal neutral sterols followed by C-3 and CD hamsters. Attention has been paid on not only total amount of cholesterol intake but also the intake modes if the data can be extrapolated to humans.

The underlying mechanism by which the frequent cholesterol intake increased the cholesterol absorption remains unclear. The cholesterol absorption is governed by two types of transporters namely NPC1L1 and ABCG 5/8. 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, the real time PCR analyses demonstrated that mRNA

NPC1L1 in 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 C-3 and C-1 groups, suggesting that other factors may also be involved in the regulation of cholesterol absorption. In this regard, the two enzymes namely ACAT2 and MTP are also essential in regulation of cholesterol absorption with the former promoting intracellular sterol esterification in the enterocytes, while MTP being responsible for the chylomicron assembly (Sudhop et al., 2005). Results showed 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 C-3 and C-1 groups, the intestinal ACAT activity in C-1 group was reduced by 52.8% compared with that in C-3 hamsters, suggesting that the frequent cholesterol intake increased the intestinal ACAT activity without necessarily up-regulating its mRNA level. Similarly, MTP had greater protein masses in CD and C-3 groups than that in C-1. The present study clearly demonstrated that the cholesterol intake frequency affected the 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. Firstly, cholesterol can incorporate into bile fluid and eliminated as the fecal neutral sterols. Secondly, cholesterol is converted to bile acids and eliminated as the fecal bile acids. Our data demonstrated that the 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 (Figure 3.3). However, CD hamsters accumulated most cholesterol in the liver followed by C-3 and C-1 groups in a decreasing order (Figure 3.2) with down-regulation of LDLR (Figure 3.3). The

present study confirmed the phenomenon that the inverse relationship between the hepatic cholesterol and LDLR number. The lower hepatic cholesterol in C-1 group led to an up-regulation of LDLR (Goldstein et al., 1987).

In conclusion, the present study was the first time to demonstrate for a given amount of dietary cholesterol, the 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 (Figure 3.6).

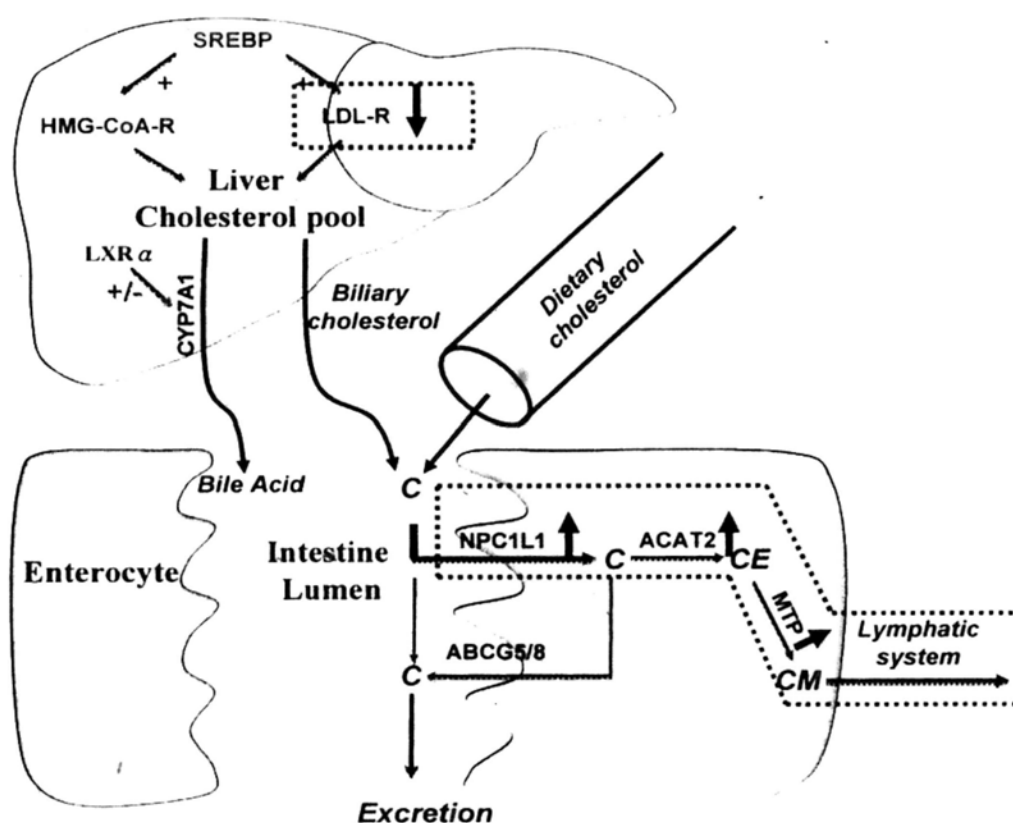


Figure 3.6 Effect of high frequency intake of cholesterol on cholesterol homeostasis

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. Also, higher frequency intake of cholesterol could reduce LDLR mRNA level (Modified from Figure 1.3).

Chapter 4

Frequent Myristic Acid Intake Is Associated with Elevation of Plasma Total Cholesterol

4.1 Introduction

SFA are claimed to elevate plasma TC and LDL levels. Myristic acid, also called tetradecanoic acid (14:0), is known to be one of the most atherogenic SFA when consumed at high levels (Fernandez et al., 2005). Myristic acid is abundantly found in nutmeg, palm kernel oil, coconut oil, butter fat while it is a minor component of many other animal fats. Besides, myristic acid is found in a significant level in most mammalian milk and is mainly in the sn-2 position on the TG molecule (Jensen et al., 1990). Average intake of myristic acid levels are about 1 g/d in Japan, 6 g/d in the United States, 8 g/d in the Netherlands, and 14 g/d in eastern Finland (Zock et al., 1994). However, no data are available on consumption of myristic acid by Chinese.

As a key component of high saturated fats in Western diet, myristic acid has been associated with an increased incidence of several chronic conditions, such as obesity, cancer, coronary artery disease and diabetes (Burdock et al., 2007). A fat rich in myristic acid given to a large number of volunteers in a well-controlled trial showed that myristic acid was about 1.5 times potent in raising cholesterol level as palmitic acid (Zock et al., 1994 & Hegsted et al., 1965 & Mensink et al., 1992). Current US dietary guidelines recommend that the intake of saturated fat be less than 10% of total caloric intake in an overall balanced daily diet (Burdock et al., 2007). Two previous studies have shown that myristic acid could induce an elevation in

plasma TC and LDL levels in both animals and humans (Hegsted et al., 1965 & Hayes et al., 1992). In addition, a linear regression was found between the percentage of myristic acid in the diets and the plasma LDL-C concentrations (Loison et al., 2002).

4.2 Objective

Previous investigations have found that dietary myristic acid increases both plasma LDL-C and HDL-C concentrations. However, no study to date has investigated the relationship between the myristic acid intake frequency and plasma lipoprotein profiles and how myristic acid intake frequency interacts with the genes of these transporters, proteins and enzymes involved in regulation of cholesterol homeostasis. The present study was therefore carried out for the first time to (i) examine the effect of myristic acid intake frequency on the gene expression of intestinal NPC1L1, ABCG 5 and 8, ACAT2 and MTP; and to (ii) characterize how myristic acid intake frequency interacts with hepatic SREBP-2, HMG-CoA-R, LDLR, CYP7A1, scavenger receptor B type I (SR-BI) and LCAT in hamsters.

4.3 Materials and methods

4.3.1 Diets

All diets were prepared as previously described (Lam et al., 2008). A myristic acid-free diet was prepared by mixing the following ingredients (g/kg diet): cornstarch, 508; casein, 242; olive oil, 50; sucrose, 119; mineral mix, 40; vitamin mix, 20; DL-methionine, 1, cholesterol, 1. A 2.5% myristic acid diet was prepared by adding 2.5% myristic acid by weight into the myristic acid-free diet. 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 −20°C.

4.3.2 Animals

Male adult Golden Syrian hamsters (*Mesocricetus auratus*; n=33, 134±18 g) were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. 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) and housed in wire-bottomed cages at 23°C in an animal room with 12-h light–dark cycle. All the hamsters were adapted to gavage-feeding 3 times of 0.7 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, one group was maintained on a 2.5% myristic acid diet (MD) throughout the entire study (high myristic acid intake frequency). It was found that MD hamsters consumed about 8.5 g of foods daily which contained about 210 mg of myristic acid. The other two groups were fed a myristic acid-free diet with a gavage-administration of 210 mg of myristic acid per day. For the regular

myristic acid intake frequency group (M-3), hamsters were daily gavage-administered 70 mg myristic acid in 0.7 ml of 3% starch solution for three times at 9:00 am, 4:00 pm, 10:00 pm, respectively, when were the three peak times of food consumption by hamsters. For the low myristic acid intake frequency group (M-1), hamsters were daily gavage-administered 210 mg myristic acid in 0.7 ml of 3% starch solution for one time; for the other two times, M-1 group was orally given 0.7 ml of 3% starch solution containing no myristic acid. Similarly, MD group was also gavage-administered three times 0.7 ml of 3% starch solution containing no myristic acid. 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 week 3 and 6 following food deprivation for 14 h and light anaesthesia, using a mixture of ketamine, xylazine and saline (v/v/v, 4:1:5). The blood was centrifuged at 1,000 × *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 the hamsters were kept for 3 days to allow for recovery and then euthanized by carbon dioxide suffocation without food deprivation. Liver, heart, kidney, adipose tissues (perirenal and epididymal pads) were removed, washed in saline, and weighed. The first 5 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.

4.3.3 Determination of plasma lipoproteins

The analyses were similarly conducted as previously described in section 2.3.4,

Chapter 2.

4.3.4 Measurement of organ cholesterol and fecal neutral and acidic sterols

Materials and experimental procedures were described in section 2.3.5 and 2.3.6, Chapter 2.

4.3.5 Western blotting analysis of liver SREBP-2, LDLR, HMG-CoA-R and CYP7A1

The analysis were similarly conducted as previously described in section 2.3.7, chapter 2 except that data on abundance of SREBP-2, LDLR, HMG-CoA-R, LXRα and CYP7A1 were normalized with β-actin (Santa Cruz Biotechnology, Inc., California, USA) not β-tubulin.

4.3.6 Real-time PCR analysis of mRNA for liver SREBP-2, LDLR, HMG-CoA-R, CYP7A1, SR-BI, LCAT and small intestine NPC1L1, ABCG5, ABCG8, ACAT2, MTP

The analysis were similarly conducted as previously described in section 3.3.6, chapter 3 except that two more genes SR-BI and LCAT were examined. SR-BI and LCAT were important in regulation of lipid metabolism (Dorfman et al., 2005 & Field et al., 2003 & Guo et al., 2001 & Zhang et al., 2004).

Gene	Accession number	Forward primer 5′ → 3′	Reverse primer 5′ →3′	TaqMan probe 5′
SR-BI	U11453	CGTGGTCCTCCCATT GCT	CTGCGTGTAGAACGT GTTCAG	CCGCTCTGCTC AAACC
LCAT	AJ275582	TTTGGCAAGACCTAT TCTGTTGAGT	CGCACATACCCATTG TTAACCAGAT	CAGTGTGTGCA TGTATGCTA

4.3.7 Measurement of intestinal ACAT activity and Western blotting analysis of intestinal MTP

Materials and experimental procedures were described in section 3.3.7, Chapter 3.

4.3.8 Statistics

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

4.4 Results

4.4.1 Food intake, body and organ weights

No significant differences in the final body weight were seen among the three groups. Similarly, there were no significant differences in food intakes among the three groups. The ratio of liver, heart, epididymal fat and perirenal fat to the final body weight was similar among the three groups (Table 4.1).

4.4.2 Serum TC, HDL-C, non-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 4.2). At the end of week 3, plasma TC and HDL-C of MD were significantly higher than that of the other two groups. At the end of week 6, plasma TC, HDL-C and Non-HDL-C demonstrated a decreasing trend among MD, M-3 and M-1 groups, and significant differences were even found between M-3 and M-1 in TC and HDL-C level. And plasma TG level of MD was higher than that of the other two groups at the end of week 6. To be specific, plasma TC levels were 275.38,

267.29 and 234.82 mg/dl, plasma HDL-C levels were 140.24, 140.62 and 126.88 mg/dl, whereas plasma Non-HDL-C were 135.14, 126.67 and 107.94 mg/dl in MD, M-3 and M-1 groups, respectively.

4.4.3 Organ cholesterol

No differences in heart, kidney and peripheral cholesterol content were seen among the three groups. In contrast, cholesterol content in the liver demonstrated an increasing trend in the MD, M-3 and M-1 groups (Figure 4.1).

4.4.4 Cholesterol balance and excretion of fecal neutral and acidic sterols

Concentration of individual neutral and acidic sterols in the feces of the hamsters at week 1, 3, 6 is shown in Table 4.3. The statistical analysis did not find any differences in the fecal output of total neutral and acidic sterols.

Total intake of cholesterol was compared with its excretion in neutral and acidic sterols (Table 4.4). It was found that no significant differences among the three groups in net cholesterol retention. The apparent cholesterol absorption was calculated by an equation [(cholesterol intake – excretion of neutral and acidic sterols)] cholesterol intake]. It was clear that the myristic acid intake frequency had no effect on the apparent cholesterol absorption.

4.4.5 Immunoblot and mRNA analyses of liver SREBP-2, LDLR, HMG-CoA-R, CYP7A1, SR-B1 and LCAT

The Western blot and real time PCR analyses demonstrated that frequency of myristic acid intake had no effect on hepatic LDLR and LCAT in hamsters (Figure 4.2 and Figure 4.3). However, real time PCR analysis demonstrated that the myristic acid in diet (MD group) was associated with lower expression of mRNA SREBP-2,

HMG-CoA-R and SR-B1. Besides, both western blot and real time PCR analysis showed that the increasing myristic acid intake frequency could increase CYP7A1 protein mass and mRNA level.

4.4.6 mRNA analyses of intestinal ABCG5, ABCG8, NPC1L1, MTP and ACAT2

The real time PCR analysis demonstrated that frequency of myristic acid intake had no effect on intestinal ABCG5, ABCG8, MTP and ACAT2. However, the NPC1L1 mRNA level demonstrated a decreased trend among MD, M-3 and M-1 groups, and significant difference was found between M-D and M-1 group (Figure 4.4).

4.4.7 Intestinal ACAT activity and Immunoblot analysis of intestinal MTP

No significant differences were found in the intestinal ACAT activity and protein mass of MTP among MD, M-3 and M-1 groups (Figure 4.5).

Table 4.1 Body weight, organ relative weight and food intake of hamsters fed the 2.5% myristic acid (MD), no myristic acid diet with gavage-administration of 70 mg myristic acid 3 times per day (M-3) and no myristic acid diet with gavage-administration of 210 mg myristic acid one time per day (M-1).

	MD	M-3	M-1
Initial body weight (g)	133.64 ± 16.58	133.45 ± 17.35	134.27 ± 22.08
Final body weight (g)	138.45 ± 12.63	134.55 ± 13.92	134.40 ± 19.33
Food intake (g/day)	8.50 ± 0.31	8.08 ± 0.42	8.30 ± 0.45
Liver/BW (%)	4.08 ± 0.39	4.16 ± 0.35	4.10 ± 0.27
Heart/BW (%)	0.36 ± 0.03	0.36 ± 0.02	0.36 ± 0.04
Kidney/BW (%)	0.75 ± 0.07	0.77 ± 0.07	0.79 ± 0.08
Epididymal fat/BW (%)	1.51 ± 0.39	1.38 ± 0.33	1.37 ± 0.26
Perirenal fat/BW(%)	0.67 ± 0.31	0.57 ± 0.21	0.54 ± 0.21

Values are expressed as mean ± S.D. (n=11).

Table 4.2 Changes in plasma TC, TG, HDL-C, Non-HDL-C in hamsters fed the 2.5% myristic acid (MD), no myristic acid diet with gavage-administration of 70 mg myristic acid 3 times per day (M-3) and no myristic acid diet with gavage-administration of 210 mg myristic acid one time per day (M-1)[1].

	MD	M-3	M-1
Week 0			
TC (mg/dL)	162.85 ± 33.60	165.39 ± 31.73	164.97 ± 35.64
HDL-C (mg/dL)	81.24 ± 10.54	83.63 ± 11.68	85.80 ± 13.38
Non-HDL-C(mg/dL)	81.61 ± 25.45	81.76 ± 23.52	79.16 ± 25.78
Non-HDL-C/HDL-C	0.99 ± 0.24	0.97 ± 0.23	0.91 ± 0.26
TG (mg/dL)	72.05 ± 23.70	77.95 ± 32.65	69.55 ± 33.14
Week 3			
TC (mg/dL)	249.93 ± 22.79^{a}	205.31 ± 26.60^{b}	222.71 ± 22.67^{b}
HDL-C (mg/dL)	127.80 ± 9.28^{a}	111.54 ± 9.51^{b}	117.64 ± 8.85^{b}
Non-HDL-C(mg/dL)	122.13 ± 17.25^{a}	93.77 ± 18.35^{b}	105.07 ± 20.98^{ab}
Non-HDL-C/HDL-C	0.96 ± 0.12^{a}	0.84 ± 0.11^{b}	0.90 ± 0.19^{ab}
TG (mg/dL)	82.17 ± 32.30	66.08 ± 23.24	66.67 ± 22.43
Week 6			
TC (mg/dL)	275.38 ± 26.73^{a}	267.29 ± 24.93^{a}	234.82 ± 20.28^{b}
HDL-C (mg/dL)	140.24 ± 9.97^{a}	140.62 ± 6.25^{a}	126.88 ± 8.96^{b}
Non-HDL-C(mg/dL)	135.14 ± 21.36^{a}	126.67 ± 25.30^{ab}	107.94 ± 16.09^{b}
Non-HDL-C/HDL-C	0.96 ± 0.14	0.90 ± 0.19	0.85 ± 0.13
TG (mg/dL)	99.04 ± 38.57^{a}	68.38 ± 16.15^{b}	70.99 ± 18.12^{ab}

[1]Values are means ± SD, n=10. [a,b] Means in a row for a given week with different superscript letters differ significantly, $p < 0.05$.

Table 4.3 Change in fecal output of individual neutral and acidic sterols in hamsters fed the 2.5% myristic acid (MD), no myristic acid diet with gavage-administration of 70 mg myristic acid 3 times per day (M-3) and no myristic acid diet with gavage-administration of 210 mg myristic acid one time per day (M-1)[1], respectively

	MD	**M-3**	**M-1**
Week 1			
Neutral sterols (mg/day)			
Coprostanol	0.59 ± 0.45	0.53 ± 0.10	0.50 ± 0.20
Cholesterol	0.18 ± 0.06	0.19 ± 0.04	0.22 ± 0.14
Dihydrocholesterol	0.12 ± 0.04	0.12 ± 0.02	0.12 ± 0.04
Campersterol	0.19 ± 0.07	0.23 ± 0.06	0.18 ± 0.06
β-sitosterol	0.04 ± 0.01	0.05 ± 0.02	0.07 ± 0.04
Total	1.13 ± 0.58	1.12 ± 0.15	1.09 ± 0.33
Acidic sterols (mg/day)			
Lithocholic acid	1.17 ± 0.58	1.16 ± 1.02	0.96 ± 0.36
Deoxycholic acid	0.18 ± 0.08[ab]	0.15 ± 0.02[b]	0.23 ± 0.04[a]
Chenodeoxycholic acid + Cholic acid	0.43 ± 0.22	0.30 ± 0.19	0.30 ± 0.15
ursodeoxycholic acid	0.20 ± 0.09	0.11 ± 0.10	0.10 ± 0.06
Total	1.98 ± 0.90	1.72 ± 1.32	1.59 ± 0.57
Week 3			
Neutral sterols (mg/day)			
Coprostanol	0.81 ± 0.15	0.72 ± 0.20	0.82 ± 0.44
Cholesterol	0.58 ± 0.20[b]	0.92 ± 0.22[a]	0.63 ± 0.22[ab]
Dihydrocholesterol	0.28 ± 0.08	0.26 ± 0.05	0.25 ± 0.04
Campersterol	0.37 ± 0.11	0.30 ± 0.07	0.32 ± 0.08
β-sitosterol	0.19 ± 0.10	0.30 ± 0.07	0.21 ± 0.07
Total	2.24 ± 0.34	2.50 ± 0.21	2.24 ± 0.57
Acidic sterols (mg/day)			
Lithocholic acid	0.99 ± 0.61	1.41 ± 0.95	1.02 ± 0.81
Deoxycholic acid	0.15 ± 0.06	0.18 ± 0.11	0.17 ± 0.17
Chenodeoxycholic acid + Cholic acid	0.25 ± 0.10	0.30 ± 0.14	0.28 ± 0.34
ursodeoxycholic acid	0.12 ± 0.07	0.17 ± 0.10	0.14 ± 0.14
Total	1.52 ± 0.81	2.07 ± 1.24	1.62 ± 1.23

Week 6	MD	M-3	M-1
Neutral sterols (mg/day)			
Coprostanol	0.83 ± 0.09	1.10 ± 0.38	1.05 ± 0.45
Cholesterol	0.70 ± 0.20	0.83 ± 0.33	0.48 ± 0.29
Dihydrocholesterol	0.26 ± 0.05	0.28 ± 0.02	0.27 ± 0.07
Campersterol	0.38 ± 0.08	0.37 ± 0.09	0.38 ± 0.08
β-sitosterol	0.13 ± 0.02	0.18 ± 0.06	0.13 ± 0.05
Total	2.29 ± 0.16	2.76 ± 0.52	2.32 ± 0.90
Acidic sterols (mg/day)			
Lithocholic acid	0.91 ± 0.68	0.51 ± 0.18	0.54 ± 0.18
Deoxycholic acid	0.12 ± 0.08	0.09 ± 0.05	0.09 ± 0.03
Chenodeoxycholic acid + Cholic acid	0.28 ± 0.13	0.21 ± 0.09	0.16 ± 0.05
ursodeoxycholic acid	0.10 ± 0.05	0.09 ± 0.03	0.09 ± 0.02
Total	1.41 ± 0.91	0.90 ± 0.28	0.88 ± 0.14

[1]Values are means ± SD, n=11. [a,b] Means in a row for a given week with different superscript letters differ significantly, $p < 0.05$.

Table 4.4 Changes in daily total cholesterol intake (mg), fecal excretion of total neutral sterols (mg), total acidic sterols (mg), and cholesterol balance in hamsters fed the 2.5% myristic acid (MD), no myristic acid diet with gavage-administration of 70mg myristic acid 3 times per day (M-3) and no myristic acid diet with gavage-administration of 210mg myristic acid one time per day (M-1)[1], respectively

	MD	M-3	M-1
Week1			
Neutral sterol (mg)	1.13 ± 0.58	1.12 ± 0.15	1.09 ± 0.33
Acidic sterol (mg)	1.98 ± 0.90	1.72 ± 1.32	1.59 ± 0.57
Cholesterol intake (mg)	8.25 ± 0.17	8.53 ± 2.11	8.20 ± 1.05
Cholesterol retained (mg)	5.14 ± 1.52	5.70 ± 2.75	5.52 ± 1.48
Cholesterol retain/cholesterol intake (%)	62.13 ± 18.02	64.87 ± 21.92	66.72 ± 12.32
Week 3			
Neutral sterol (mg)	2.24 ± 0.34	2.50 ± 0.21	2.24 ± 0.57
Acidic sterol (mg)	1.52 ± 0.81	2.07 ± 1.24	1.62 ± 1.23
Cholesterol intake (mg)	9.07 ± 0.17	9.05 ± 1.54	9.08 ± 1.18
Cholesterol retained (mg)	5.31 ± 0.96	4.48 ± 2.20	5.22 ± 2.02
Cholesterol retain/cholesterol intake (%)	58.67 ± 11.10	48.21 ± 15.95	56.26 ± 17.57
Week 6			
Neutral sterol (mg)	2.29 ± 0.16	2.76 ± 0.52	2.32 ± 0.90
Acidic sterol (mg)	1.41 ± 0.91	0.90 ± 0.28	0.88 ± 0.14
Cholesterol intake (mg)	9.40 ± 0.79	8.56 ± 0.95	8.74 ± 1.11
Cholesterol retained (mg)	5.70 ± 0.32	4.90 ± 1.00	5.54 ± 1.02
Cholesterol retain/cholesterol intake (%)	61.09 ± 7.39	57.01 ± 6.41	63.39 ± 9.62

[1]Values are means ± SD, n=11.

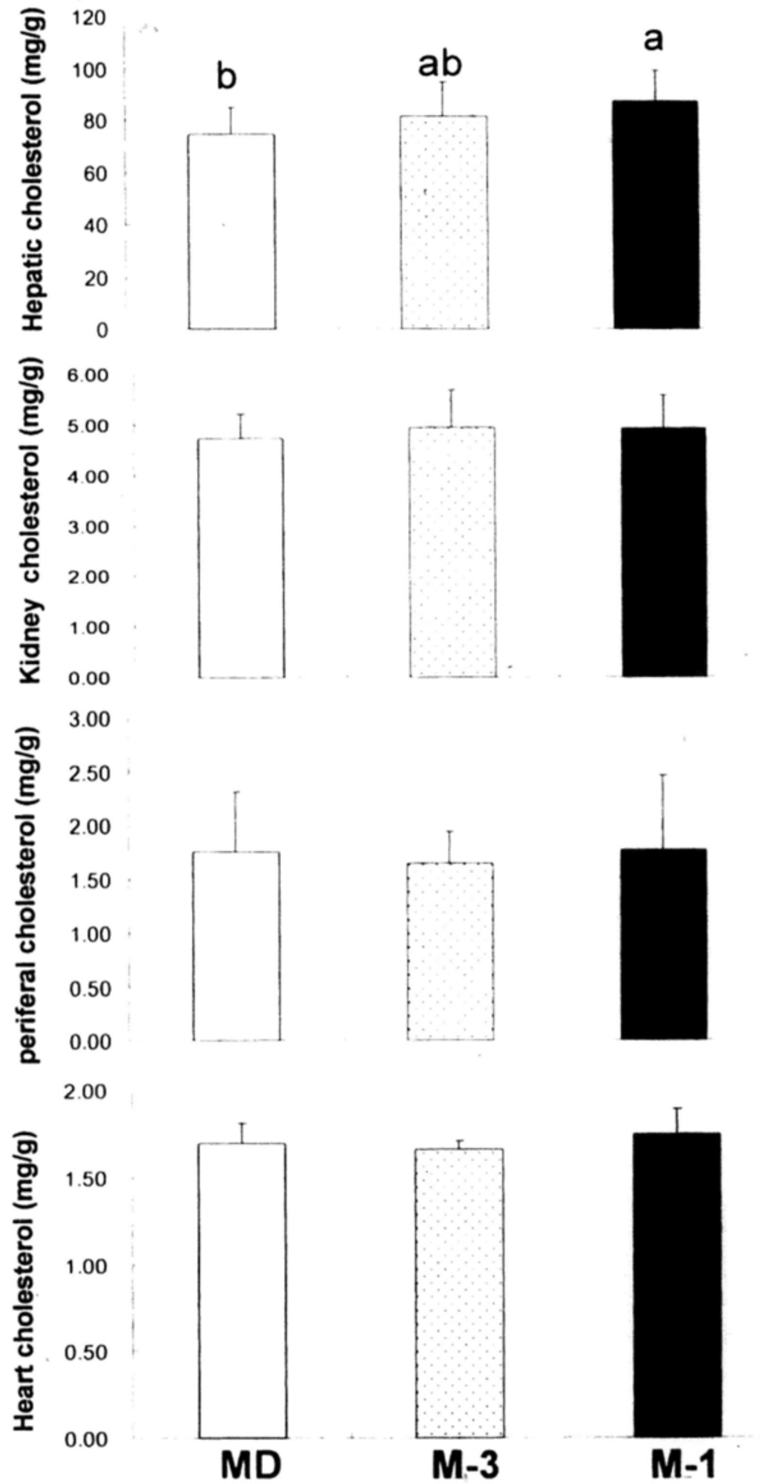


Figure 4.1

Effect of frequent myristic acid intake on cholesterol content in the liver, adipose tissue, kidney and heart. [a,b]Means with different letters differ significantly, $p<0.05$

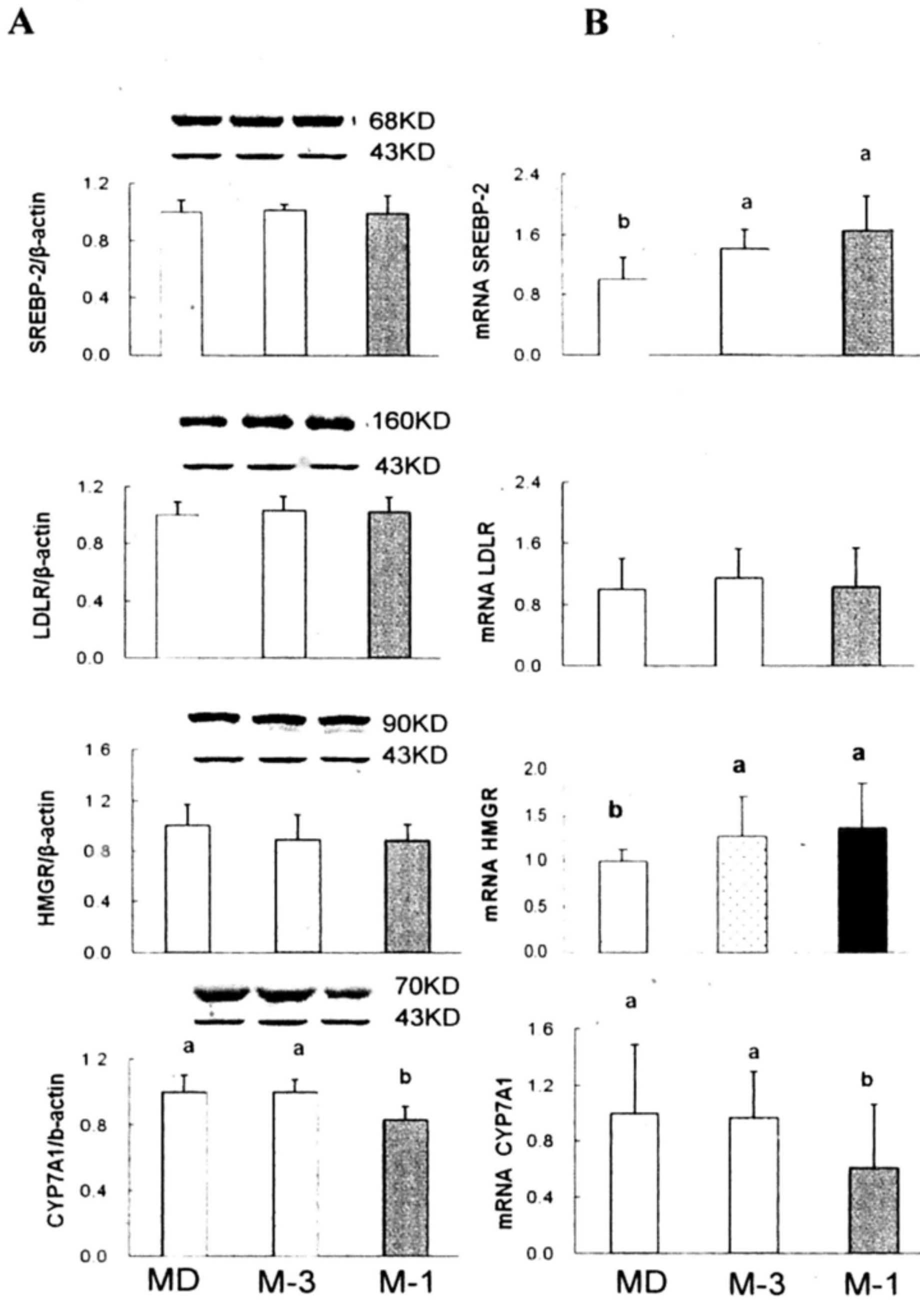


Figure 4.2

The relative immunoreactive mass (A) and mRNA (B) of hepatic SREBP-2, HMG-CoA-R, LDLR and CYP7A1 in hamsters fed the 2.5% myristic acid (MD), no myristic acid diet with gavage-administration of 70 mg myristic acid 3 times per day (M-3) and no myristic acid diet with gavage- administration of 210 mg myristic acid one time per day (M-1). Values are expressed as means ± SD (n=11) with those for MD group being arbitrarily taken as one. [a,b]Means with different superscrip letters differ significantly, $p<0.05$.

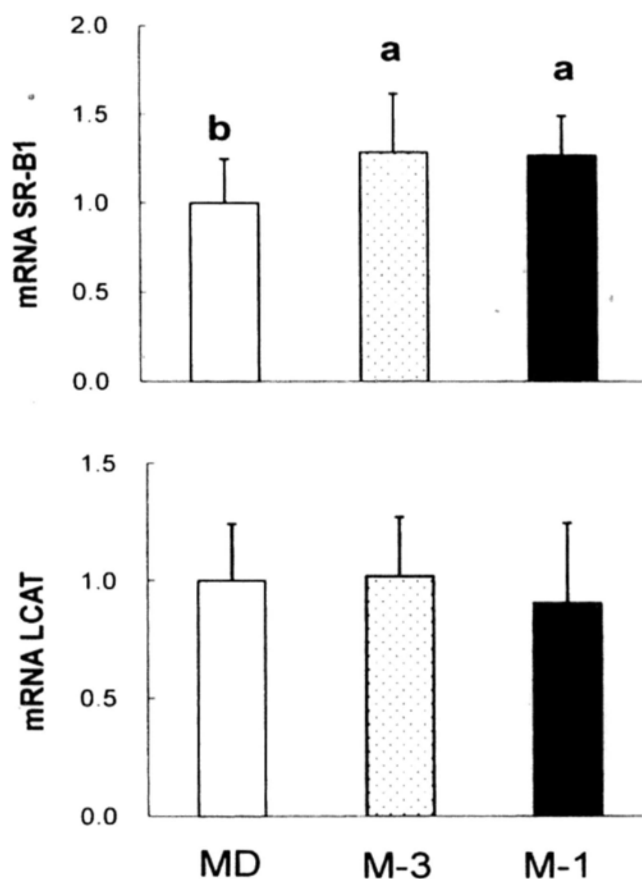


Figure 4.3

The mRNA of hepatic SR-BI and LCAT in hamsters fed the 2.5% myristic acid (MD), no myristic acid diet with gavage-administration of 70 mg myristic acid 3 times per day (M-3) and no myristic acid diet with gavage- administration of 210 mg myristic acid one time per day (M-1). Values are expressed as means ± SD (n=11) with those for MD group being arbitrarily taken as one. [a,b]Means with different superscrip letters differ significantly, $p<0.05$.

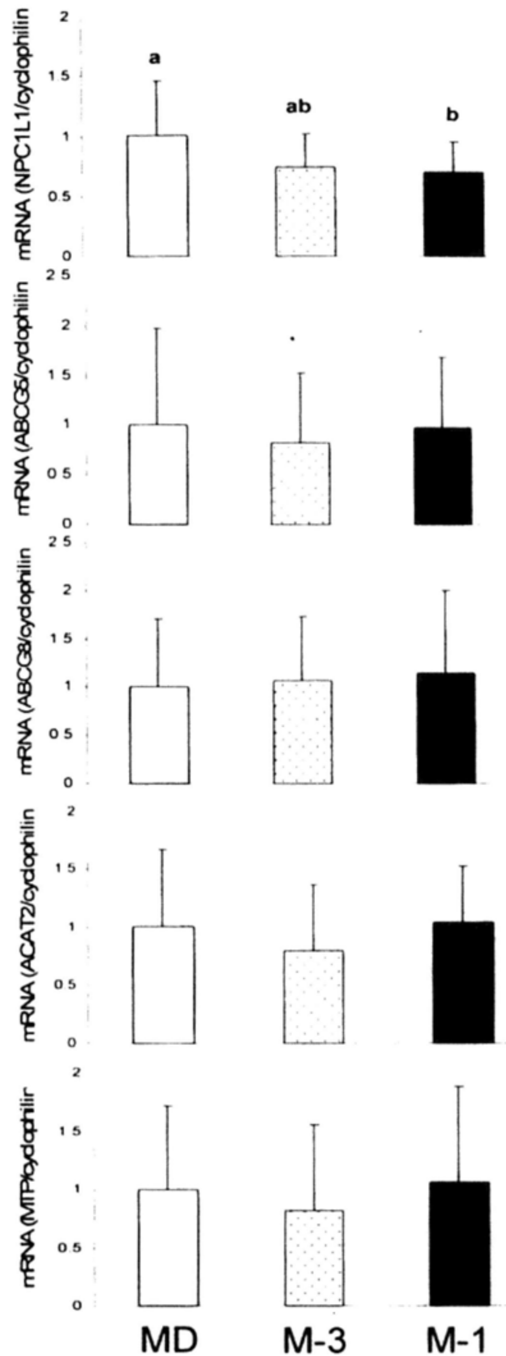


Figure 4.4

The relative mRNA of intestinal NPC1L1, ABCG5/8, ACAT2 and MTP in hamsters fed the 2.5% myristic acid (MD), no myristic acid diet with gavage-administration of 70 mg myristic acid 3 times per day (M-3) and no myristic acid diet with gavage-administration of 210 mg myristic acid one time per day (M-1). Values are expressed as means ± SD (n=11) with those for MD group being arbitrarily taken as one. [a,b]Means with different superscrip letters differ significantly, $p<0.05$.

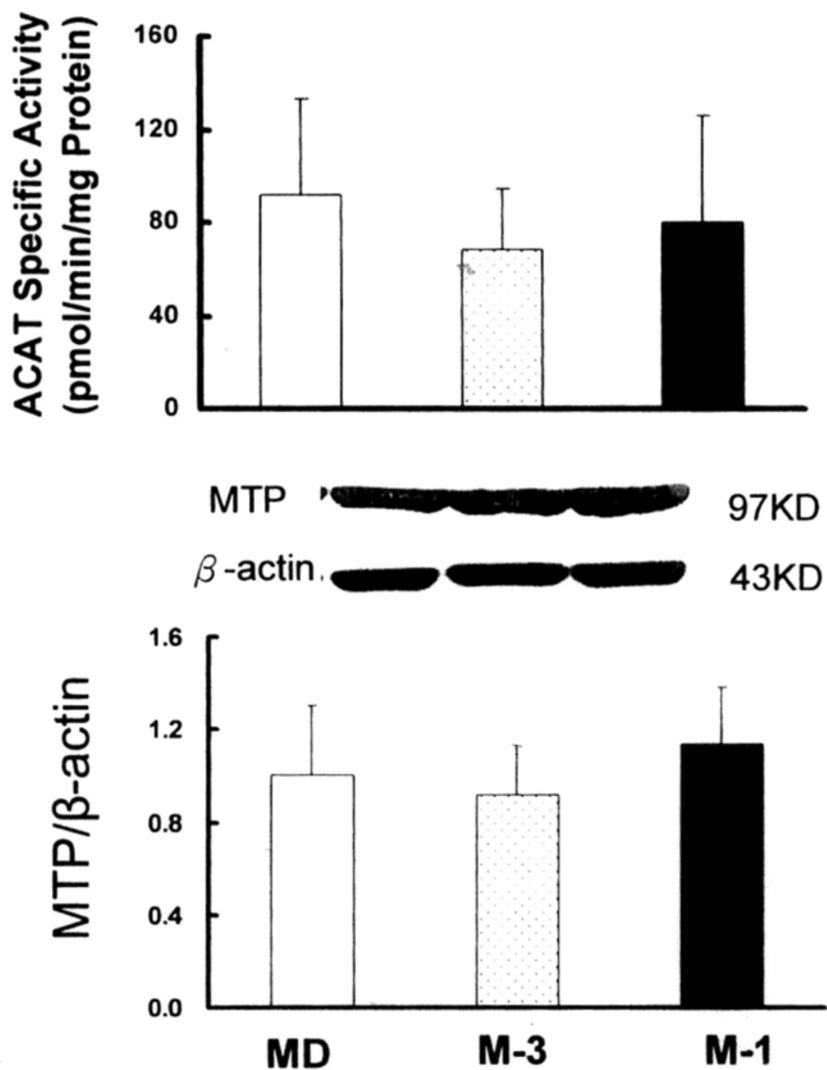


Figure 4.5

Intestinal ACAT activity and Immunoblot analyses of intestinal MTP in hamsters fed the 2.5% myristic acid (MD), no myristic acid diet with gavage-administration of 70 mg myristic acid 3 times per day (M-3) and no myristic acid diet with gavage-administration of 210 mg myristic acid one time per day (M-1).

4.5 Discussion

In the present study, hamsters were given daily 210 mg of myristic acid either in diet (MD) or a gavage-administration of 3 times 70 mg (M-3) and 1 time 210 mg (M-1). Results demonstrated clearly that there was an increasing trend in concentrations of plasma TC and non-HDL-C in association with the myristic acid intake frequency at the end of week 6 (Table 4.2). Plasma HDL-C of M-1 group was much lower than MD and M-3 groups. The underlying mechanism by which the frequent myristic acid intake increased the plasma TC and non-HDL-C remains unclear. Maintaining cholesterol level in the blood is in fact a complex process of transport of cholesterol and lipids into and out of various compartments: (1) from the gut, liver or periphery into the circulation; (2) from the circulation into the periphery and the liver; (3) cholesterol de novo synthesis of in the liver; and (4) excretion from the liver as bile acids (Lichtenstein, 2008). It deems necessary to target the interaction of myristic acid intake frequency with these genes which were involved in these processes were examined in our study.

The fecal sterol analysis showed that there were no differences in fecal total neutral or acidic sterols outputs (Table 4.3). As the change in cholesterol absorption generally affects fecal sterol concentrations (Chen et al., 2006 & Chevallier et al., 1973 & Lutton et al., 1980), the fecal sterol output data implied that myristic acid intake frequency had no effect on cholesterol absorption. We hypothesize that there be some kind of relationship between cholesterol transporters and fatty acid absorption. Thus, the gene expression of intestinal cholesterol transporters was examined by real time PCR analysis. In fact, the mRNA level of NPC1L1 demonstrated a decreasing trend among MD, M-3 and M-1 groups, and significant difference was found between MD and M-1 (Figure 4.4). However, there were no differences among the three groups in ABCG5, ABCG8, ACAT2 and MTP gene

expression. It was truly hard to explain the underlying mechanism that the frequency of myristic acid intake only affected intestinal NPC1L1 mRNA level in hamsters in our study. However, a recent study could provide an indirect evidence that the NPC1L1 pathway could affect dietary fat absorption, primarily long-chain saturated fatty acids, possibly by influencing the amount of fatty acid transport protein 4 (FATP4) present in enterocyte membranes (Labonte´ et al., 2008). Fatty acid transport proteins form a family of six related proteins that are highly conserved during evolution with representatives in all vertebrate and invertebrate species as well as in yeast (Stahl et al., 2004 & Pohl et al., 2004 & Doege et al., 2006). And all mammalian FATPs have been reported to increase fatty acid uptake upon over expression (Stahl et al., 2004 & Doege et al., 2006). Although, we didn't examine the FATPs gene expression, the present study might imply that the frequency of myristic acid intake could affect fatty acid absorption in the small intestine, and NPC1L1 might be involved in this process.

Since the liver is the main organ concerned with the regulation of cholesterol homeostasis, the major hepatic activities implicated in this process have to be assayed in order to better understand the role of myristic acid consumption frequency. The mRNA level of SREBP-2 and HMG-CoA-R demonstrated an increasing trend among MD, M-3 and M-1 groups, and significant differences were found in both SREBP-2 and HMG-CoA-R mRNA level between MD and the other two groups (Figure 4.2). It is known that SREBP-2 responsive genes in the cholesterol biosynthetic pathway include HMG-CoA synthase, HMG-CoA-R, farnesyl diphosphate synthase, and squalene synthase (Horton et al., 2002). In this regard, HMG-CoA-R is a rate limiting enzyme in cholesterol de novo synthesis. The observed increasing trend in the mRNA HMG-CoA-R could be explained by the increasing trend in hepatic cholesterol concentration among MD, M-3 and M-1

groups (Figure 4.1). However, it is still unknown why the myristic acid intake frequency had no effect on SREBP-2 and HMG-CoA-R protein amount. Also, we found that there was an increasing trend in both transcriptional and translational levels of CYP7A1 in association with the myristic acid intake frequency (Figure 4.2). Dietary myristic acid could modulate bile acid biosynthesis which is the major process of cholesterol degradation in humans and animals (Vlahcevic et al., 1996). Cheema et al (2000) had also demonstrated that fatty acids could stimulate the murine CYP7A1 and human CYP7A1 gene promoters via PPARα/RXRα. Thus, the frequent myristic acid intake could increase bile acid excretion in the liver. Like the LDL receptor in humans, SR-BI is regarded as an HDL receptor and plays a major role in cholesterol regulation, especially in rodents (Combettes-Souverain et al., 1999). In our study, the hepatic expression of the SR-BI in MD group was the lowest among the three groups (Figure 4.3). In addition, plasma HDL-C of MD and M-3 group was significantly higher than that of ML group (Table 4.2), this is in agreement with the results of Loison et al (2002), who demonstrated that increasing the amount of myristic acid in the diet was the most important factor in the increase of HDL-C concentration and this effect was linked to a decrease in the amount of SR-BI in the liver.

In conclusion, the present study was the first time to demonstrate for a given amount of dietary myristic acid, the increasing consumption frequency elevated plasma TC, non-HDL-C, HDL-C and decreased hepatic cholesterol levels. Elevation of plasma TC and HDL-C is most likely mediated by up-regulation of NPC1L1 and down-regulation of SR-BI gene expression via enhancement of dietary myristic acid absorption. In addition, the decline of hepatic cholesterol level among MD, M-3 and M-1 groups is probably regulated by down-regulation of SREBP-2 and HMG-CoA-R gene expression.

Chapter 5
Higher Frequency of Beta-Sitosterol Intake Has no Effect on Plasma Cholesterol

5.1 Introduction

Interest in cholesterol-lowering activity of phytosterols is growing. Over 40 phytosterols have been identified with β-sitosterol, campesterol, and stigmasterol being most abundant in nature. A major health application is to add these phytosterols into fats and vegetable oils as a cholesterol-lowering functional ingredient. It has been shown that a high intake of dietary phytosterols is inversely related to serum TC and LDL concentrations (Jones et al., 1997; Katan et al., 2003; Andersson et al., 2004), and the daily recommended consumption is 2 g (Law, 2000). The mechanism underlying the hypocholesterolemic activity of phytosterols is to inhibit the intestinal absorption of cholesterol (Plat et al., 2005). Phytosterols are poorly absorbed in the intestine. It is estimated only about 5% of β-sitosterol, 15% of campesterol, and less than 1% of dietary stanols are absorbed (Jones et al., 1997). In contrast, more than 50% dietary cholesterol can be absorbed. The differential absorption rate of cholesterol and phytosterols in the intestine can explain why phytosterol could reduce cholesterol absorption and have no atherogenic effect. Firstly, phytosterols compete with cholesterol for NPC1L1, limiting the entry of cholesterol into enterocytes. Once some phytosterols enter the enterocytes, they are largely prevented from being further absorbed, because ABCG5 and ABCG8 return them to the lumen of the intestine (Graf et al., 2002). Secondly, ACAT2 prefers to esterify cholesterol to phytosterols, and effectively eliminates phytosterols from the absorption process (Temel et al., 2003).

5.2 Objective

To date, only two human studies had examined the effect of phytosterol consumption frequency on plasma lipoprotein profiles, but the results turned out differently (Plat et al., 2000 & AbuMweis et al., 2009). It is unknown of how phytosterol consumption frequency interacts with the genes and proteins involved in lipoprotein metabolism *in vivo*. The present study was therefore carried out for the first time to (i) examine the effect of β-sitosterol intake frequency on the gene expression of intestinal NPC1L1, ABCG 5 and 8, ACAT2 and MTP; and to (ii) characterize how β-sitosterol intake frequency interacts with hepatic SREBP-2, HMG-CoA-R, LDLR and CYP7A1 in hamsters.

5.3 Materials and methods

5.3.1 Diets

The basal diet was prepared by mixing the following ingredients (g/kg diet) as previously described (Lam et al., 2008): cornstarch, 508; casein, 242; lard, 50; sucrose, 119; mineral mix, 40; vitamin mix, 20; DL-methionine, 1. The powered diets were mixed with a gelatin solution (20 g/L) in a ratio of 200 g diet per liter. Once the gelatin had set, the diets were cut into pieces of approximately 10 g cubes and stored frozen at −20°C.

5.3.2 Animals

Male adult Golden Syrian hamsters (n=33, 125±8 g) were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. 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) and housed in wire-bottomed cages at 23°C in an animal room with 12-h light–dark cycle. All the hamsters were adapted to gavage-feeding 3 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, all the hamsters were maintained on the basal diet throughout the entire study. For the control group, hamsters were daily gavage-administration 3 mg cholesterol in 0.5 ml 3% starch solution for three times at 9:00 am, 4:00 pm, 10:00 pm, respectively. For the high β-sitosterol intake frequency group (S-3), hamsters were daily gavage-administration 3 mg β-sitosterol with 3 mg cholesterol in 0.5 ml of 3% starch solution for three times. For the low β-sitosterol intake frequency group (S-1), hamsters were daily gavage-administration

9 mg β-sitosterol and 3 mg cholesterol in 0.5 ml 3% starch solution for one time; for the other two times, S-1 group was gavage-administration 3 mg cholesterol in 0.5 ml of 3% starch solution. β-sitosterol was purchased from Sino-Future Pharmaceutical Company (Xi'an, China). 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 week 3 and 6 following food deprivation for 14 h and light anaesthesia, 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 the hamsters were kept for 3 days to allow for recovery and then euthanized by carbon dioxide suffocation without food deprivation. Liver, heart, kidney, adipose tissues (perirenal and epididymal pads) were removed, washed in saline, and weighed. The first 5 cm of duodenum was discarded, and the following 30 cm of the small intestine was collected. All tissue samples were flash frozen in liquid nitrogen and stored at −80°C until analysis.

5.3.3 Determination of plasma lipoproteins

The analyses were similarly conducted as previously described in section 2.3.4, Chapter 2.

5.3.4 Measurement of liver, plasma sterols and fecal neutral and acidic sterols

Materials and experimental procedures were described in section 2.3.5 and 2.3.6, Chapter 2. Particularly, plasma sterols were extracted with addition of 50 ug 5α-cholestane as an internal standard (200 ul for the plasma sample) using the same

method as the fecal neutral sterol analysis.

5.3.5 Western blotting analysis of liver SREBP-2, LDLR, HMG-CoA-R and CYP7A1

The analysis were similarly conducted as previously described in section 2.3.7, Chapter 2 except that data on abundance of SREBP-2, LDLR, HMG-CoA-R and CYP7A1 were normalized with β-actin (Santa Cruz Biotechnology, Inc., California, USA) not β-tubulin.

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

The analysis was similarly conducted as previously described in section 3.3.6.

5.3.7 Statistics

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

5.4 Results

5.4.1 Effect of β-sitosterol consumption frequency on food intake, body and organ weights

No significant differences in the final body weight were seen among the three groups. Similarly, there were no significant differences in food intakes among the three groups. The ratio of liver, heart, kidney, epididymal fat and perirenal fat to the final body weight was similar among the three groups (Table 5.1).

5.4.2 Effect of β-sitosterol consumption frequency serum TC, HDL-C, non-HDL-C, TG and HDL-C/TC

There was no difference in plasma lipoprotein profiles among the three groups at week 0 (Table 5.2). At the end of week 2, plasma TC and Non-HDL-C of the control group were significantly higher than those of S-3 and S-1 groups. And HDL-C/TC ratio of S-1 and S-3 groups was significantly higher than that of the control group (Table 5.2). At the end of week 4, plasma TC of the control group was still significantly higher than the other two groups, but there was no significant difference between S-3 and S-1 groups. Similarly, S-1 and S-3 groups had lower TC and Non-HDL-C level but higher HDL-C/TC ratio than the control group. No differences were found between S-3 and S-1 groups at the end of week 6. To be specific, plasma TC levels were 253.68, 229.93 and 213.98 mg/dl, plasma Non-HDL-C levels were 139.53, 119.20 and 105.93 mg/dl, and HDL-C/TC ratios were 0.45, 0.48 and 0.51 in the control, S-3 and S-1 groups, respectively. The β-sitosterol intake frequency had no effect on plasma TG level during the entire experiment (Table 5.2).

5.4.3 Effect of β-sitosterol consumption frequency liver and plasma sterols

In the liver, S-1 and S-3 groups could decrease hepatic cholesterol level. However, significant difference was only found between S-1 and the control group (Table 5.3). Hepatic stigmasterol of the control group was higher than that of S-3 and S-1 groups, but no differences were found in liver β-sitosterol and campersterol among the three groups. Plasma β-sitosterol level of S-1 group was significantly higher than that of S-3 and the control groups (Table 5.3). Plasma campersterol level of the control groups was higher than S-3 and S-1 groups, and significant difference was found between control and S-3 groups. There were no significant differences in the plasma stigmasterol levels among the three groups.

5.4.4 Effect of β-sitosterol consumption frequency cholesterol balance and excretion of fecal neutral and acidic sterols

The individual neutral and acidic sterols in the feces at week 1, 4, 6 were quantified (Table 5.4). In general, S-3 and S-1 groups had significantly higher fecal dihydrocholesterol, campersterol, ursodeoxycholic acid and fecal total neutral sterols than the control group. Also, the β-sitosterol excretion was much higher in S-3 and S-1 groups compared with the control group. To be specific, at week 6, total fecal neutral sterol level was 3.47, 5.36 and 5.85 mg/day, ursodeoxycholic acid levels were 0.05, 0.38 and 0.45mg/d in the control, S-3 and S-1 groups, respectively. Fecal β-sitosterol output of S-3 and S-1 groups was 84 and 93 fold higher than that of the control group. The statistical analysis did not find any difference in the fecal total acidic sterol output.

Total intake of cholesterol was compared with its excretion in neutral and acidic sterols (Table 5.5). 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 control group was much higher than that of S-3

and S-1 groups, but no difference was found between S-3 and S-1 groups. The apparent cholesterol absorption was calculated by an equation [(cholesterol intake – excretion of neutral and acidic sterols)]/ cholesterol intake]. It was clear that β-sitosterol intake could reduce the apparent cholesterol absorption, but the increasing β-sitosterol intake frequency had no effect.

5.4.5 Effect of β-sitosterol consumption frequency immunoblot analyses of liver SREBP-2, LDLR, HMG-CoA-R, and CYP7A1

The western blot analyses demonstrated that β-sitosterol intake had no effect on the liver HMG-CoA-R, LDLR, SREBP-2 and CYP7A1 in hamsters among the three groups (Figure 5.1).

5.4.6 Effect of β-sitosterol consumption frequency mRNA analyses of intestinal ABCG5, ABCG8, NPC1L1, MTP and ACAT2

Real time PCR analyses demonstrated that except for ACAT2, the gene expression of NPC1L1, ABCG5, ABCG8 and MTP in S-1 and S-3 groups was significantly up-regulated compared with that of the control group, but no differences were found between S-1 and S-3 groups (Figure 5.2).

Table 5.1 Body weight, organ relative weight and food intake in hamsters fed the basal diet with a gavage-administration of 3 mg cholesterol 3 times per day (control), or a gavage-administration of 3 mg β-sitosterol with 3 mg cholesterol 3 times per day (S-3), or a gavage-administration of 9 mg β-sitosterol with 3 mg cholesterol for one time and 3mg cholesterol for the other two times per day (S-1).

	Control	S-3	S-1
Initial body weight (g)	125.00 ± 9.49	124.55 ± 6.22	125.08 ± 7.24
Final body weight (g)	132.73 ± 6.75	132.00 ± 10.51	130.67 ± 7.44
Food intake (g/day)	10.41 ± 0.39	10.40 ± 0.42	10.29 ± 0.40
Liver/BW (%)	5.33 ± 0.35	5.04 ± 0.39	5.16 ± 0.48
Heart/BW (%)	0.35 ± 0.03	0.34 ± 0.02	0.36 ± 0.02
Kidney/BW (%)	0.85 ± 0.06	0.86 ± 0.05	0.88 ± 0.04
Epididymal fat/BW (%)	2.03 ± 0.34	2.10 ± 0.30	1.97 ± 0.30
Perirenal fat/BW (%)	1.02 ± 0.24	1.15 ± 0.22	0.98 ± 0.20

Values are expressed as mean ± S.D. (n=11).

Table 5.2 Changes in plasma TC, TG, HDL-C, Non-HDL-C and HDL-C/TC ratio in hamsters fed the basal diet with a gavage-administration of 3 mg cholesterol 3 times per day (control), or a gavage-administration of 3 mg *β*-sitosterol with 3 mg cholesterol 3 times per day (S-3), or a gavage-administration of 9 mg *β*-sitosterol with 3 mg cholesterol for one time and 3 mg cholesterol for the other two times per day (S-1).

	Control	S-3	S-1
Week 0			
TC (mg/dL)	130.74 ± 15.73	130.91 ± 7.37	130.10 ± 18.57
HDL-C (mg/dL)	71.99 ± 7.28	71.90 ± 3.29	71.79 ± 4.06
Non-HDL-C(mg/dL)	58.75 ± 13.54	59.01 ± 6.90	58.32 ± 19.44
HDL-C/TC	0.56 ± 0.06	0.55 ± 0.03	0.56 ± 0.10
TG (mg/dL)	171.58 ± 70.18	161.82 ± 39.56	161.22 ± 64.21
Week 2			
TC (mg/dL)	223.13 ± 19.50^{a}	199.09 ± 23.24^{b}	193.07 ± 33.36^{b}
HDL-C (mg/dL)	108.93 ± 9.51	109.93 ± 9.43	103.57 ± 12.19
Non-HDL-C(mg/dL)	114.20 ± 14.47^{a}	89.16 ± 19.23^{b}	89.51 ± 23.80^{b}
HDL-C/TC	0.49 ± 0.04^{b}	0.56 ± 0.05^{a}	0.54 ± 0.05^{a}
TG (mg/dL)	182.64 ± 67.71	155.39 ± 48.00	165.66 ± 65.79
Week 4			
TC (mg/dL)	228.68 ± 23.34^{a}	206.00 ± 23.80^{ab}	198.50 ± 30.40^{b}
HDL-C (mg/dL)	111.66 ± 8.62^{a}	107.72 ± 8.53^{ab}	101.42 ± 10.26^{b}
Non-HDL-C(mg/dL)	117.02 ± 18.53	98.28 ± 21.54	97.09 ± 24.25
HDL-C/TC	0.49 ± 0.04	0.53 ± 0.07	0.52 ± 0.06
TG (mg/dL)	204.94 ± 79.92	171.23 ± 58.22	190.00 ± 68.39
Week 6			
TC (mg/dL)	253.68 ± 21.65^{a}	229.93 ± 25.43^{b}	213.98 ± 33.71^{b}
HDL-C (mg/dL)	114.16 ± 8.49	110.73 ± 10.65	108.05 ± 11.67
Non-HDL-C(mg/dL)	139.53 ± 15.62^{a}	119.20 ± 16.77^{b}	105.93 ± 23.75^{b}
HDL-C/TC	0.45 ± 0.02^{b}	0.48 ± 0.03^{a}	0.51 ± 0.04^{a}
TG (mg/dL)	201.19 ± 48.14	168.73 ± 60.94	178.25 ± 41.04

Data are expressed as mean ± SD, n=11. ^{a,b}Means in a row with different letters differ significantly, $p < 0.05$.

Table 5.3 Liver and plasma (week 6) sterol contents of hamsters fed the basal diet with a gavage-administration of 3 mg cholesterol 3 times per day (control), or a gavage-administration of 3 mg β-sitosterol with 3 mg cholesterol 3 times per day (S-3), or a gavage-administration of 9 mg β-sitosterol with 3 mg cholesterol for one time and 3 mg cholesterol for the other two times per day (S-1).

	Control	S-3	S-1
Liver			
Cholesterol (mg/g)	35.27 ± 6.49^{a}	32.93 ± 4.40^{ab}	29.61 ± 6.32^{b}
Campersterol (mg/g)	0.08 ± 0.04	0.07 ± 0.02	0.06 ± 0.02
Stigmasterol (mg/g)	0.25 ± 0.05^{a}	0.21 ± 0.02^{b}	0.21 ± 0.03^{b}
β-sitosterol (mg/g)	0.09 ± 0.06	0.09 ± 0.04	0.09 ± 0.05
Plasma (week 6)			
Cholesterol (mg/dL)	253.68 ± 21.65^{a}	229.93 ± 25.43^{b}	213.98 ± 33.71^{b}
Campesterol (mg/ dL)	0.22 ± 0.13	0.26 ± 0.12	0.27 ± 0.12
Campesterol/Cholesterol ($X 10^{-3}$)	0.88 ± 0.19^{b}	1.13 ± 0.24^{a}	1.26 ± 0.32^{a}
Stigmasterol (mg/ dL)	1.30 ± 0.42	1.16 ± 0.23	1.32 ± 0.29
β-sitosterol (mg/ dL)	0.71 ± 0.46^{b}	0.84 ± 0.18^{b}	1.20 ± 0.45^{a}

Data are expressed as mean ± SD, n=11. [a,b]Means in a row with different letters differ significantly, $p < 0.05$.

Table 5.4 Change in fecal output of individual neutral and acidic sterols in hamsters fed the basal diet with a gavage-administration of 3 mg cholesterol 3 times per day (control), or a gavage-administration of 3 mg β-sitosterol with 3 mg cholesterol 3 times per day (S-3), or a gavage-administration of 9 mg β-sitosterol with 3 mg cholesterol for one time and 3mg cholesterol for the other two times per day (S-1).

	Control	S-3	S-1
Week 1			
Neutral sterols (mg/day)			
Coprostanol	0.99 ± 0.28	1.04 ± 0.19	1.07 ± 0.18
Coprostanone	0.08 ± 0.02^{a}	0.05 ± 0.02^{b}	0.04 ± 0.01^{b}
Cholesterol	1.89 ± 0.42	2.32 ± 0.19	2.16 ± 0.31
Dihydrocholesterol	0.17 ± 0.03^{b}	0.30 ± 0.02^{a}	0.31 ± 0.02^{a}
Campersterol	0.06 ± 0.01^{b}	1.48 ± 0.08^{a}	1.49 ± 0.09^{a}
Total	3.19 ± 0.40^{b}	5.19 ± 0.29^{a}	5.06 ± 0.52^{a}
β-sitosterol	0.01 ± 0.01^{c}	2.97 ± 0.23^{a}	2.39 ± 0.25^{b}
Acidic sterols (mg/day)			
Lithocholic acid	1.04 ± 0.33^{a}	0.55 ± 0.21^{b}	0.54 ± 0.20^{b}
Deoxycholic acid	0.19 ± 0.11	0.15 ± 0.10	0.24 ± 0.23
Chenodeoxycholic acid + Cholic acid	0.21 ± 0.09	0.23 ± 0.08	0.22 ± 0.09
ursodeoxycholic acid	0.13 ± 0.05^{b}	0.65 ± 0.32^{a}	0.69 ± 0.29^{a}
Total	1.58 ± 0.51	1.58 ± 0.63	1.70 ± 0.68
Week 4			
Neutral sterols (mg/day)			
Coprostanol	0.72 ± 0.16^{b}	1.20 ± 0.33^{a}	0.82 ± 0.17^{ab}
Coprostanone	0.03 ± 0.01^{a}	0.01 ± 0.00^{b}	0.02 ± 0.01^{ab}
Cholesterol	2.78 ± 0.60	2.34 ± 0.38	2.49 ± 0.39
Dihydrocholesterol	0.26 ± 0.03^{b}	0.39 ± 0.04^{a}	0.33 ± 0.02^{ab}
Campersterol	0.09 ± 0.02^{b}	1.58 ± 0.25^{a}	1.34 ± 0.18^{a}
Total	3.88 ± 0.63^{b}	5.53 ± 0.52^{a}	4.99 ± 0.46^{a}
β-sitosterol	0.07 ± 0.05^{b}	3.41 ± 0.49^{a}	3.20 ± 0.30^{a}

Acidic sterols (mg/day)	**Control**	**S-3**	**S-1**
Lithocholic acid	0.67 ± 0.44	0.39 ± 0.22	0.43 ± 0.06
Deoxycholic acid	0.09 ± 0.05	0.08 ± 0.06	0.10 ± 0.04
Chenodeoxycholic acid + Cholic acid	0.25 ± 0.14	0.24 ± 0.08	0.32 ± 0.09
ursodeoxycholic acid	0.08 ± 0.04[b]	0.27 ± 0.18[a]	0.53 ± 0.27[a]
Total	1.09 ± 0.52	0.98 ± 0.93	1.38 ± 0.32
Week 6			
Neutral sterols (mg/day)			
Coprostanol	0.51 ± 0.08[b]	0.88 ± 0.41[ab]	0.98 ± 0.25[a]
Coprostanone	0.02 ± 0.01[b]	0.03 ± 0.01[a]	0.04 ± 0.02[ab]
Cholesterol	2.64 ± 0.56	2.62 ± 0.51	2.91 ± 0.16
Dihydrocholesterol	0.23 ± 0.02[b]	0.33 ± 0.07[a]	0.36 ± 0.04[a]
Campersterol	0.07 ± 0.00[b]	1.51 ± 0.48[a]	1.56 ± 0.11[a]
Total	3.47 ± 0.59[b]	5.36 ± 0.48[a]	5.85 ± 0.46[a]
β-sitosterol	0.04 ± 0.01[b]	3.37 ± 0.71[a]	3.73 ± 0.15[a]
Acidic sterols (mg/day)			
Lithocholic acid	0.63 ± 0.21	0.66 ± 0.39	0.67 ± 0.48
Deoxycholic acid	0.12 ± 0.03	0.12 ± 0.08	0.13 ± 0.10
Chenodeoxycholic acid + Cholic acid	0.11 ± 0.04[b]	0.38 ± 0.25[a]	0.25 ± 0.11[a]
ursodeoxycholic acid	0.05 ± 0.02[b]	0.38 ± 0.22[a]	0.45 ± 0.31[a]
Total	0.91 ± 0.23	1.55 ± 1.39	1.50 ± 0.98

Data are expressed as mean ± SD, n=11. [a,b]Means in a row with different letters differ significantly, $p < 0.05$.

Table 5.5 Changes in daily total cholesterol intake (mg), fecal excretion of total neutral sterols (mg), total acidic sterols (mg), and cholesterol balance in hamsters fed the basal diet with a gavage-administration of 3 mg cholesterol 3 times per day (control), or a gavage-administration of 3 mg *β*-sitosterol with 3 mg cholesterol 3 times per day (S-3), or a gavage-administration of 9 mg *β*-sitosterol with 3 mg cholesterol for one time and 3mg cholesterol for the other two times per day (S-1).

	Control	**S-3**	**S-1**
Week1			
Neutral sterol (mg)	3.19 ± 0.40 [b]	5.19 ± 0.29 [a]	5.06 ± 0.52 [a]
Acidic sterol (mg)	1.58 ± 0.51	1.58 ± 0.63	1.70 ± 0.68
Cholesterol intake (mg)	9.00 ± 0.00	9.00 ± 0.00	9.00 ± 0.00
Cholesterol retained (mg)	4.23 ± 0.63 [a]	2.23 ± 0.37 [b]	2.25 ± 1.14 [b]
Cholesterol retain/cholesterol intake (%)	47.03 ± 6.97 [a]	24.74 ± 4.14 [b]	24.97 ± 12.69 [b]
Week 4			
Neutral sterol (mg)	3.88 ± 0.63 [b]	5.53 ± 0.52 [a]	4.99 ± 0.46 [a]
Acidic sterol (mg)	1.09 ± 0.52	0.98 ± 0.93	1.38 ± 0.32
Cholesterol intake (mg)	9.00 ± 0.00	9.00 ± 0.00	9.00 ± 0.00
Cholesterol retained (mg)	4.04 ± 0.89 [a]	2.50 ± 0.50 [b]	2.63 ± 0.51 [b]
Cholesterol retain/cholesterol intake (%)	44.84 ± 9.94 [a]	27.75 ± 5.51 [b]	29.25 ± 5.62 [b]
Week 6			
Neutral sterol (mg)	3.47 ± 0.59 [b]	5.36 ± 0.48 [a]	5.85 ± 0.46 [a]
Acidic sterol (mg)	0.91 ± 0.23	1.55 ± 1.39	1.50 ± 0.98
Cholesterol intake (mg)	9.00 ± 0.00	9.00 ± 0.00	9.00 ± 0.00
Cholesterol retained (mg)	4.62 ± 0.63 [a]	2.09 ± 1.25 [b]	1.65 ± 1.05 [b]
Cholesterol retain/cholesterol intake (%)	51.35 ± 6.99 [a]	23.21 ± 13.93 [b]	18.36 ± 11.62 [b]

Data are expressed as mean ± SD, n=11. [a,b]Means in a row with different letters differ significantly, $p < 0.05$.

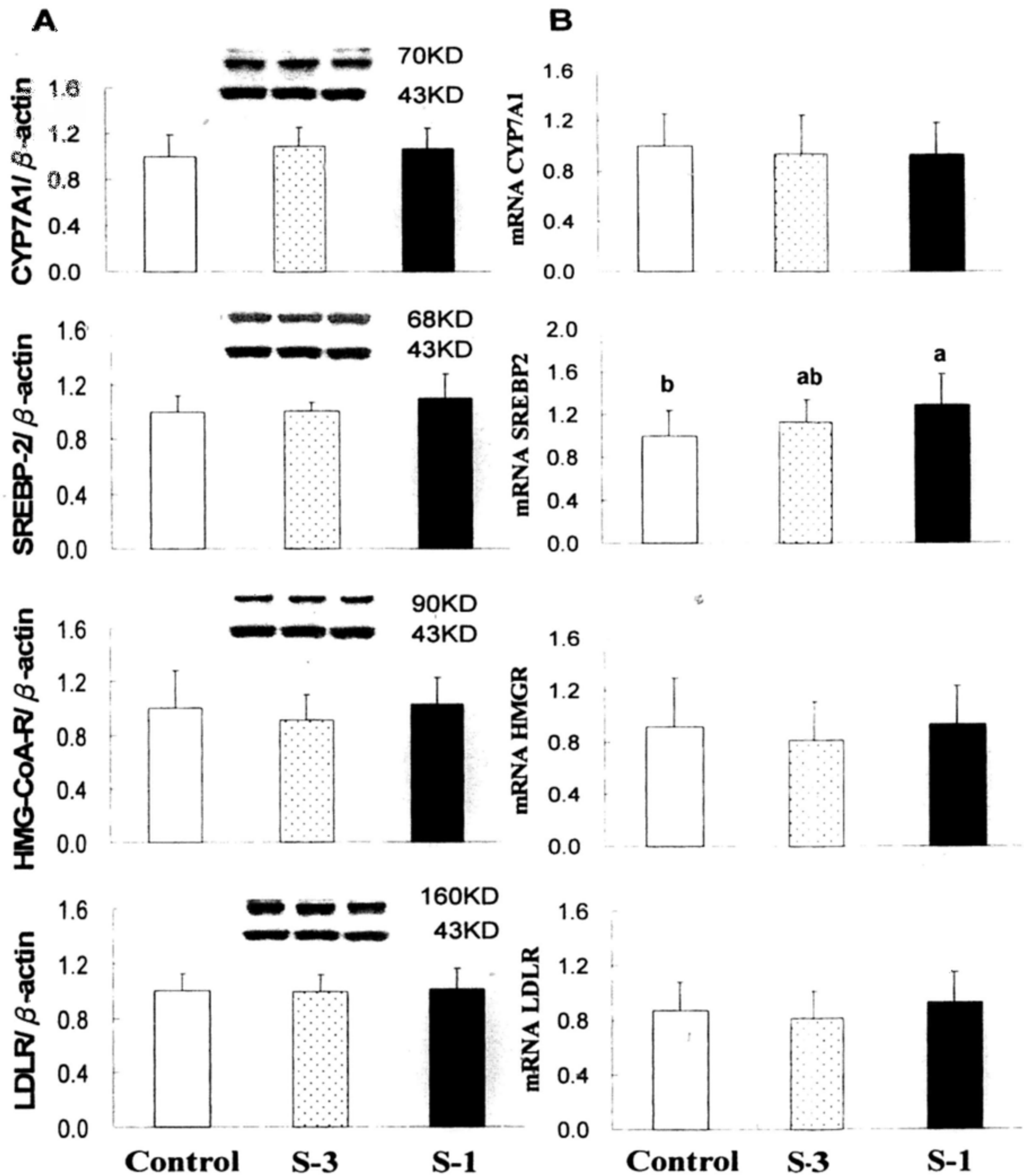


Figure 5.1 The relative immunoreactive mass (A) and mRNA (B) of hepatic SREBP-2, HMG-CoA-R, LDLR and CYP7A1 in hamsters fed the basal diet with a gavage-administration of 3 mg cholesterol 3 times per day (control), or a gavage-administration of 3 mg β-sitosterol with 3 mg cholesterol 3 times per day (S-3), or a gavage-administration of 9 mg β-sitosterol with 3 mg cholesterol for one time and 3mg cholesterol for the other two times per day (S-1). Values are expressed as means ± SD (n=11) with those for control group being arbitrarily taken as one. [a,b]Means with different superscrip letters differ significantly, $p<0.05$.

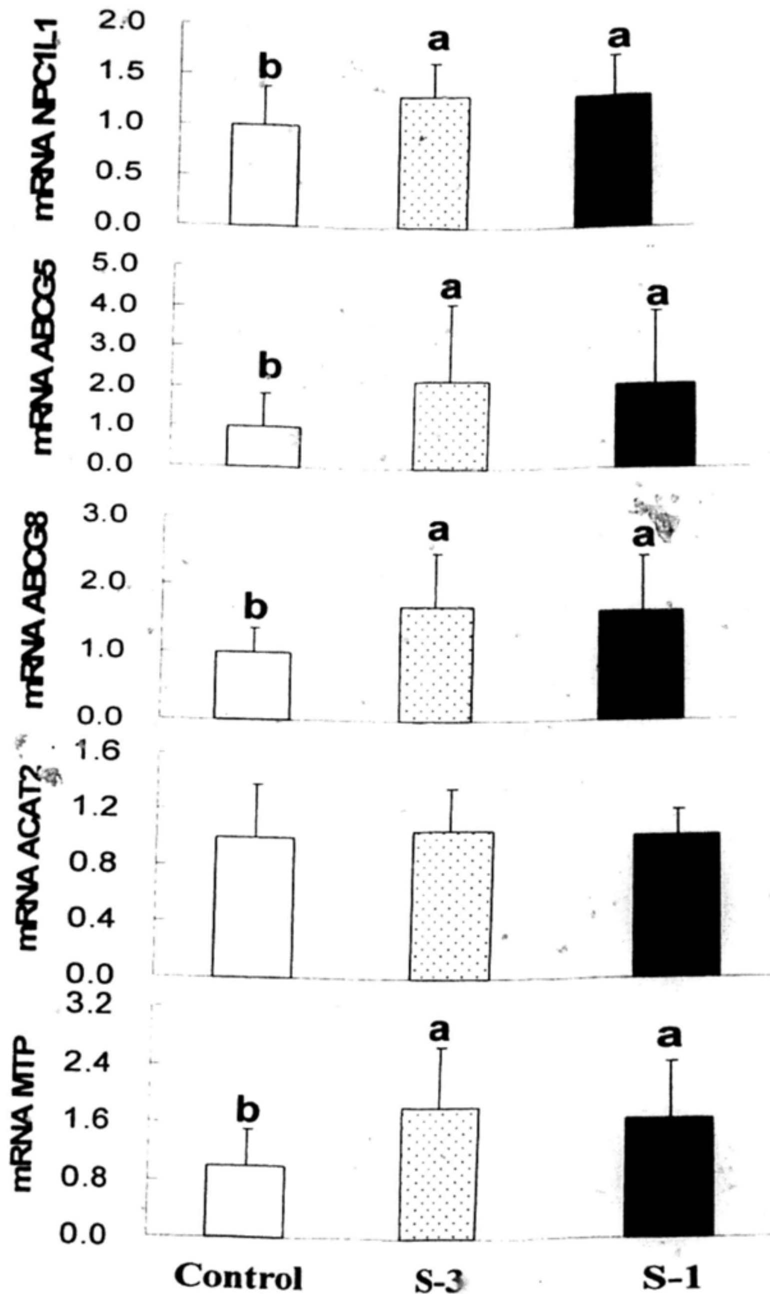


Figure 5.2 The relative mRNA of intestinal NPC1L1, ABCG5/8, ACAT2 and MTP in hamsters fed the basal diet with a gavage-administration of 3 mg cholesterol 3 times per day (control), a gavage-administration of 3 mg β-sitosterol with 3 mg cholesterol 3 times per day (S-3), or a gavage-administration 9 mg β-sitosterol with 3 mg cholesterol for one time and 3mg cholesterol for the other two times per day (S-1). Values are expressed as means ± SD (n=11) with those for control group being arbitrarily taken as one. [a,b]Means with different superscrip letters differ significantly, $p<0.05$.

5.5 Discussion

The present study demonstrated that β-sitosterol decreased, however, its intake frequency had no effect on plasma cholesterol for a given amount. Compared with the control group, hamsters gavage-given β-sitosterol had a significant decrease in plasma TC and Non-HDL-C concentration. This favorable change was associated with a significant increase in fecal excretion of coprostanol, dihydrocholesterol, campersterol, ursodeoxycholic acid and total neutral sterols ($p<0.05$; Table 5.4), suggesting β-sitosterol reduced the neutral sterol absorption. The present data did not support the claim that the increasing β-sitosterol intake frequency could be more effective in reducing plasma TC. This is reflected by the observation S-3 group had plasma TC and Non-HDL-C levels similar to those in S-1 group (Table 5.2). To take the issue further, no significant difference in the fecal sterol excretion was seen between S-3 and S-1 groups (Table 5.4). If the data can be extrapolated to humans, for a given dose, one time or three times of administration of β-sitosterol a day should have a similar cholesterol-lowering potency.

The present study found β-sitosterol decreased the hepatic cholesterol, while the increasing intake frequency had no significant effect on hepatic β-sitosterol concentration. The underlying mechanism by which β-sitosterol administration did not lead to accumulation of β-sitosterol in the liver is probably attributed to its efficient secretion into the bile (Salen et al., 1970 & Ntanios et al., 2003). However, it remains unclear the administration of β-sitosterol led to a lowered hepatic stigmasterol level compared with the control group ($p<0.05$).

The ratio of plasma campesterol to cholesterol is usually used as a biomarker for cholesterol absorption. As shown in Table 5.4, it is clearly that the administration of β-sitosterol increased this ratio, indicating that β-sitosterol decreased the cholesterol absorption. The present study investigated the interaction of β-sitosterol with the

gene expression of intestinal ACAT2, NPC1L1, ABCG5, ABCG8 and MTP. It was found that all the genes except for ACAT-2 were up-regulated. NPC1L1 is a principal intestinal transporter responsible for cholesterol and phytosterol absorption (Altmann et al., 2004 & Davis et al., 2004). The present study, in agreement with the reports of Field et al., 2004 and Chen et al., 2009, indicated that NPC1L1 might not be a target gene responsible for the cholesterol-lowering action of β-sitosterol. Instead, increased expression of NPC1L1 was probably the result of a feedback mechanism to compensate for decreased cholesterol availability caused by a competitive activity of phytosterols in micelles (Chen et al., 2009). ABCG5 and 8 preferentially pump phytosterols out of enterocytes into the lumen, resulting in limited phytosterol incorporation (Oram et al., 2006). The present results showed that β-sitosterol administration up-regulated intestinal ABCG5 and ABCG8 mRNA expression in line with the observation that fecal total neutral sterol and β-sitosterol output of S-3 and S-1 groups were much higher than those of the control group ($P<0.05$).

The real time PCR analyses demonstrated that gavage-administration of β-sitosterol did not affect ACAT2 mRNA level. Previous studies showed that sitosterol was poorly esterified by ACAT-2 (Swell et al., 1959; Bhattacharyya et al., 1979). In an *in vitro* study, Liu et al (2005) found that without cholesterol, sitosterol was a poor substrate for ACAT. Regarding the intestinal MTP, limited information is available on the relationship between phytosterol and MTP. It remains poorly understood how β-sitosterol up-regulated the gene expression of MTP.

In conclusion, the present study clearly demonstrated the cholesterol-lowering activity of β-sitosterol increased the gene expression of SREBP-2 but was not to be associated with hepatic HMG-CoA-R, LDLR and CYP7A1 in both immunoreactive mass and mRNA level (Figure 5.1), instead, was mediated by its inhibition on the

intestinal cholesterol absorption with up-regulation of NPC1L1, ABCG5, ABCG8 and MTP (Figure 5.3). For a given dose of β-sitosterol, the administration frequency had no or little effect on plasma lipoprotein profile.

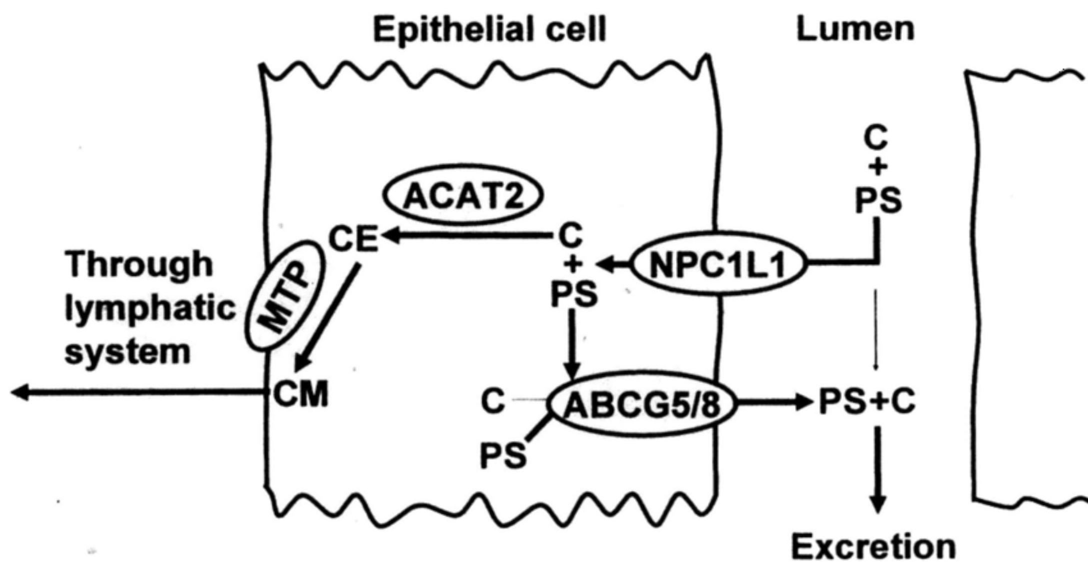


Figure 5.3 Effect of β-sitosterol intake on intestinal cholesterol absorption

Chapter 6
Conclusion

The plasma cholesterol level in particularly LDL-associated cholesterol level is one of the main risk factors of atherosclerosis. Decreasing blood TC and LDL-C levels with cholesterol-lowering nutraceuticals and dietary intake modification could slow or reverse the progression of atherosclerosis.

GSP has been shown to be hypocholesterolemic in both humans and animals. The present study affirmed dietary GSP reduced not only plasma TC and Non-HDL-C levels but also plasma TG in a dose-dependent manner. Supplementation of 1.0 % GSP for six weeks could decrease serum TC by 15% and TG by 30% in hamsters. In addition, the present study clearly demonstrated that dietary GSP was able to increase the excretion of bile acids by 3-4 folds, this was partially mediated by up-regulation of CYP7A1 in both transcriptional and translational levels. The present study also investigated the effect of dietary GSP on SREBP-2, LDLR and HMG-CoA-R, finding that GSP had no effect on their protein and mRNA levels except it increased only the mRNA HMG-CoA-R. The observed increase in the mRNA HMG-CoA-R could be explained by the decrease in cholesterol concentration in the liver of the 1.0% GSP group. It was concluded that the hypocholesterolemic activity of GSP was most likely mediated by enhancement of bile acid excretion and up-regulation of CYP7A1.

Dietary cholesterol elevates plasma TC level. In the present study, the results demonstrated clearly that there was an increasing trend in concentrations of plasma TC, Non-HDL-C, TC/HDL-C ratio and TG in association with the cholesterol intake frequency. It is the first time to demonstrate that the increasing cholesterol intake

frequency increased the apparent cholesterol absorption. This effect was reflected on the observation that C-1 group excreted most fecal neutral sterols followed by C-3 and CD hamsters. Western blotting analyses revealed that the intake frequency had no effect on protein mass of hepatic SREBP-2, LXRα, HMG-CoA-R, LDLR, and CYP7A1. However, the frequent cholesterol intake down-regulated the mRNA level of hepatic LDLR. In contrast, CD up-regulated the mRNA levels of intestinal NPC1L1, ACAT2, and MTP. Although no differences in mRNA ACAT2 and MTP were seen between C-3 and C-1 groups, the intestinal ACAT activity in C-1 group was reduced by 52.8% compared with that in C-3 hamsters, suggesting that the frequent cholesterol intake increased the intestinal ACAT activity without necessarily up-regulating its mRNA level. Similarly, MTP had greater protein masses in CD and C-3 groups than that in C-1. 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.

SFA are claimed to elevate plasma TC and LDL levels. Myristic acid is known to be one of the most atherogenic SFA when consumed at high levels. The present study demonstrated clearly that there was an increasing trend in concentrations of plasma TC and Non-HDL-C in association with the myristic acid intake frequency at the end of week 6. Plasma HDL-C of M-1 group was much lower than MD and M-3 groups. The fecal sterol analysis showed that there were no differences in fecal total neutral or acidic sterols outputs which implied that myristic acid intake frequency had no effect on cholesterol absorption. However, the mRNA level of NPC1L1 demonstrated a decreasing trend among MD, M-3 and M-1 groups, and significant difference was found between MD and M-1. A recent study found that the NPC1L1 pathway could affect dietary fat absorption, primarily long-chain saturated fatty acids, possibly by influencing the amount of FATP4 present in enterocyte

membranes. So the present study might imply that the frequency of myristic acid intake could affect fatty acid absorption in the small intestine, and NPC1L1 might be involved in this process. The mRNA level of SREBP-2 and HMG-CoA-R demonstrated an increasing trend among MD, M-3 and M-1 groups, and significant differences were found in both SREBP-2 and HMG-CoA-R mRNA level between MD and the other two groups. The observed increasing trend in the mRNA HMG-CoA-R could be explained by the increasing trend in hepatic cholesterol concentration among MD, M-3 and M-1 groups. Also, we found that there was an increasing trend in both transcriptional and translational levels of CYP7A1 in association with the myristic acid intake frequency. Thus, the frequent myristic acid intake could increase bile acid excretion in the liver. The hepatic expression of the scavenger receptor BI (SR-BI) in MD group was the lowest among the three groups which could be explained by the increase in plasma HDL-C of MD and M-3 groups. It was concluded that the myristic acid intake frequency-induced elevation of plasma TC and HDL-C is most likely mediated by up-regulation of NPC1L1 and down-regulation of SR-BI gene expression via enhancement of dietary myristic acid absorption. In addition, the decline of hepatic cholesterol level among MD, M-3 and M-1 groups is probably regulated by down-regulation of SREBP-2 and HMG-CoA-R gene expression.

Phytosterols have the cholesterol-lowering activity. The present study found that the increasing β-sitosterol intake frequency could not be more effective in reducing plasma TC. This is reflected by the observation S-3 group had plasma TC and Non-HDL-C levels similar to those in S-1 group. Compared with the control group, hamsters gavage-given β-sitosterol had a significant decrease in plasma TC and Non-HDL-C concentration. This favorable change was associated with a significant increase in fecal excretion of coprostanol, dihydrocholesterol, campersterol,

ursodeoxycholic acid and total neutral sterols, suggesting β-sitosterol reduced the neutral sterol absorption. However, no significant difference in the fecal sterol excretion was seen between S-3 and S-1 groups. The present study found β-sitosterol decreased the hepatic cholesterol, while the increasing intake frequency had no significant effect on hepatic β-sitosterol concentration. Also, it is clearly that the administration of β-sitosterol decreased the ratio of plasma campesterol to cholesterol, indicating that β-sitosterol decreased the cholesterol absorption. In addition, the present study found that the administration of β-sitosterol increased the gene expression of intestinal NPC1L1, ABCG5, ABCG8 and MTP except for ACAT-2, and had no effect on hepatic HMG-CoA-R, LDLR, SREBP-2 and CYP7A1. It was concluded that the cholesterol-lowering activity of β-sitosterol was mediated by its inhibition on the intestinal cholesterol absorption with up-regulation of NPC1L1, ABCG5, ABCG8 and MTP. However, for a given dose of β-sitosterol, the administration frequency had no or little effect on plasma lipoprotein profile.

In conclusion, the present study confirmed that hypocholesterolemic activity of GSP was most likely mediated by enhancement of bile acid excretion and up-regulation of CYP7A1. The present study also demonstrated that frequent cholesterol and myristic acid intake is associated with elevation of plasma TC level, while β-sitosterol intake frequency had no effect on plasma cholesterol for a given amount.

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