EFFECTS OF DIETARY LIPIDS ON HEPATIC CHOLESTEROL HOMEOSTASIS IN F1B HAMSTERS

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Effects of Dietary Lipids on Hepatic Cholesterol Homeostasis in F1B Hamsters

By

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ABSTRACT

The regulation of cholesterol homeostatsis is of considerable interest because of the host of studies that show a clear positive relationship between plasma cholesterol levels and the risk of coronary heart disease. It has become apparent that plasma cholesterol levels are affected by a number of life style factors including the amount and type of fat in the diet. However, the mechanisms whereby dietary fat affects plasma cholesterol remain unclear. In this study we have investigated the effects of the levels and types of dietary fat on the cholesterol homeostasis. We used the F1B strain of hamster as a model as this strain has been shown to be sensitive to dietary fat induced atherosclerosis. We propose that this sensitivity reflects differences in the regulation of lipid metabolism between the F1B and the parent strain of hamsters.

Cholesterol homeostasis in the liver is tightly regulated by several sterol-sensitive regulatory proteins and receptors, including LDL receptor, HMG-CoA reductase, ACAT, CYP7, and SREBPs. It is postulated that the ER cholesterol is the cholesterol regulatory pool plays a major role in the regulation of these enzymes of the whole cell.

We examined the effect of different dietary fats on the activity or expression of these proteins and observed that the activities of these enzymes are not directly regulated by the fats and cholesterol in the diet; instead, they appear more responsive to changes in the lipid environment of the microsome in the F1B hamster liver. Both

the HMG-CoA reductase and the ACAT activity are positively correlated with the level of n-3 fatty acids in the microsome, and ACAT activity also depend on the content of microsomal cholesterol.

We suggested that the levels of n-3 fatty acids and cholesterol in the diets may change the lipid composition microsomal membranes. This might alter membrane fluidity or the distribution of the key enzymes and regulatory proteins in the membrane of the ER. We suggest that the formation of microdomains (rafts) may sequester much of the cholesterol into pools that are separate from the cholesterol regulatory pool in the ER. However, further studies on the lipid composition of these microdomains are needed for clarify our hypothesis.

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TABLES OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACAT	acyl-coenzyme A:cholesterol acyltransferase
bHLH-Zip	basic helix-loop-helix-leucine zipper
CHD	coronary heart disease
CYP7	cholesterol 7alpha-hydroxylase
HDL	high density lipoprotein
HDL-C	high density lipoprotein-cholesterol
HMG CoA reductase	3-Hydroxymethylglutaryl Coenzyme-A reductase
LDL	low density lipoprotein
LDL-C	low density lipoprotein-cholesterol
LDL-r	low density lipoprotein-receptor
MUFA	monounsaturated fatty acid
nSREBP	nuclear form of SREBP
n-3 PUFA	omega-3 polyunsaturated fatty acid
n-6 PUFA	omega-6 polyunsaturated fatty acid
pSREBP	precursor form of SREBP
PUFA	polyunsaturated fatty acid
S1P, S2P	site-1, site-2 proteases
SFA	saturated fatty acid

SCAP	SREBP cleavage-activating protein
SREBP	sterol regulatory element binding protein
SRE	sterol regulatory element
TC	total cholesterol
VLDL	very low density lipoprotein

Chapter 1. Introduction

1.1 Lipoproteins and Coronary Heart Disease

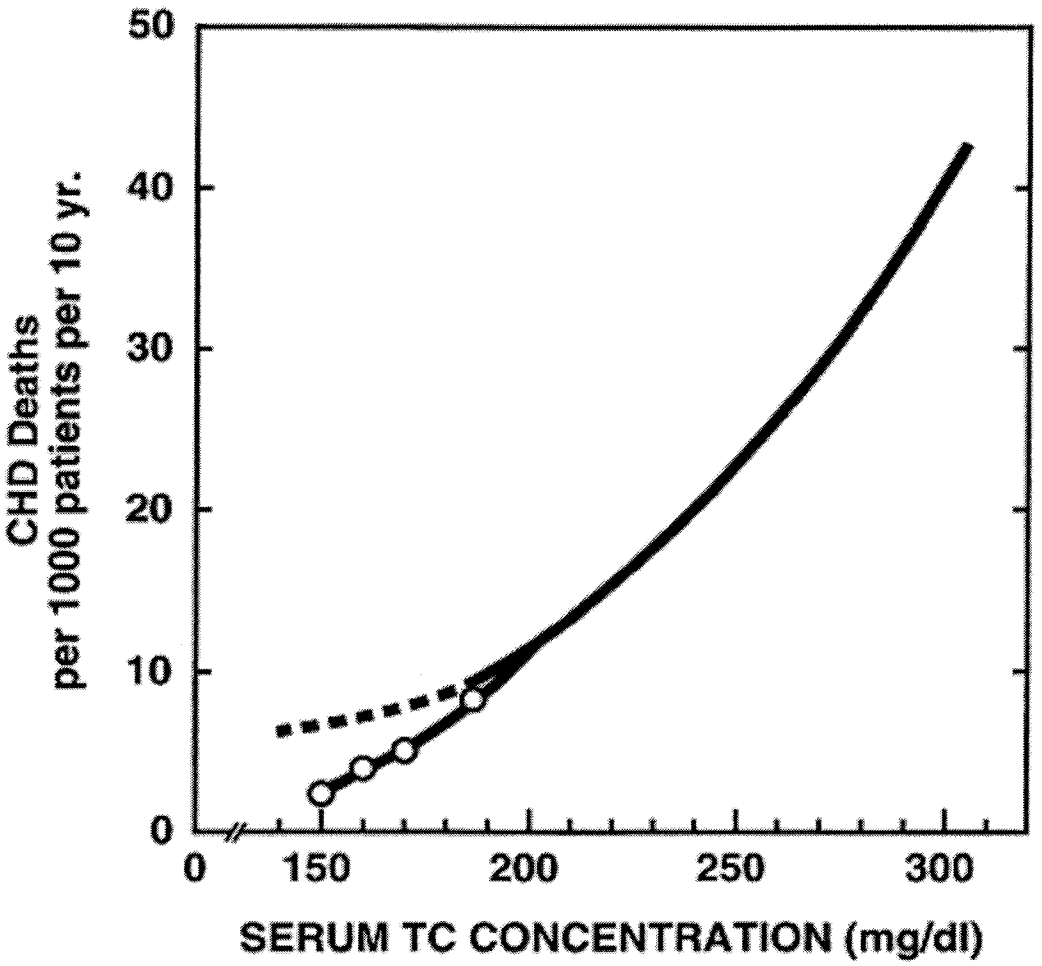
Despite extensive research for over five decades, coronary heart disease (CHD) remains a major cause of death and disability in Western countries. About 13 million Americans have CHD, 1.5 million have a myocardial infraction (MI) each year, and about 450,000 die of CHD each year (American Heart Association, 2004). For several decades there has been mounting evidence that the levels of cholesterol in the plasma can impact on the risk of developing CHD.

CHD is caused by atherosclerosis, a process characterized by endothelial dysfunction and cholesterol deposition in macrophages and smooth muscle cells in the arterial wall as a result of elevated low density lipoproteins (LDL), increased lipoprotein(a), increased remnant lipoproteins, reduced LDL receptors and decreased high density lipoproteins (HDL) . During this process, proliferation, inflammation and calcification of smooth muscle and thrombosis can occur resulting in narrowing of the arteries leading to myocardial infarction or death of heart muscle (Schaefer, 2002).

Hypercholesterolemia is critical in the development of atherosclerosis. An investigation of over 356,000 healthy males for 10 years by the National Heart, Lung, and Blood Institute (NHLBI) showed a strong correlation between the rate of CHD death and the plasma total cholesterol (TC) concentration (Figure 1.1) (1988;Stamler *et al.*, 1986). These data suggest that there is a direct relationship between the serum TC and the rate of death from CHD. It is clear from these data that death due to CHD is very uncommon in individuals with a serum TC < 3.6 mM (140mg/dl) (Dietschy, 1998). Major risk factors associated with atherosclerosis are elevated levels of plasma cholesterol, decreased levels of HDL, elevated levels of very low density lipoproteins

Figure 1.1. Mortality rates from coronary heart disease (CHD) as a function of serum total cholesterol (TC) concentrations.

These curves were constructed after recalculating data from two sources and show the number of deaths per 1000 patients per 10 years. The dashed curve comes from an analysis of nearly 356,000 men (1988;Stamler *et al.*, 1986). The four data points in the lower solid curve were recalculated from a more recent study in urban Chinese populations (Chen *et al.*, 1991).



(Dietschy, 1998)

(VLDL) plus LDL, elevated levels of triacylglycerols, and hypertension.

Since lipids, including cholesterol, are insoluble in the blood, they are transported in the plasma in particles known as lipoproteins. Among the lipoproteins, the LDL represents the major cholesterol carrying lipoprotein in human plasma and its uptake by the cells is carefully regulated (Havel, 1997). However, when LDL becomes oxidized in the arterial wall, a separate receptor pathway leads to its uncontrolled uptake by macrophages and the formation of foam cells (Krieger and Herz, 1994). In contrast, HDL appears to protect the arterial wall from the development of atherosclerosis by removing cholesterol from the intima through its role in the reverse cholesterol transport pathway (Fielding and Fielding, 1995) and by preventing the oxidation of LDL.

1.2 Dietary Fats and Plasma Lipoproteins

Over the past 25 years, epidemiological studies have shown that the level of dietary fat is positively correlated with the serum TC value and mortality from CHD (Epstein, 1989). Early studies also established that isocaloric substitution of unsaturated fats derived mostly from vegetable oil, for saturated fats from animal sources decreased the serum TC concentration in humans (Aharens, Jr., 1957; Keys *et al.*, 1957; Kinsell *et al.*, 1952). Moreover, early studies by Bang and Dyerberg demonstrated that diets rich in omega-3 polyunsaturated fatty acids (n-3 PUFA) appear to be especially effective at reducing atherogenesis (Bang *et al.*, 1971).

Initial studies suggested that the lipoprotein fraction most affected by the levels and types of dietary fats was the LDL (Aharens, Jr., 1957; Keys *et al.*, 1957; Kinsell *et al.*, 1952). However, later it was found that the levels of both atherogenic LDL-cholesterol

(LDL-C) and the anti-atherogenic HDL- cholesterol (HDL-C) are affected by dietary lipids, but the changes in HDL were modest (Lewis, 1990). Many controlled feeding studies on the effects of different dietary fatty acids on plasma cholesterol have been summarized in several meta-analyses (Hegsted *et al.*, 1965; Hegsted *et al.*, 1993; Hu and Willett, 2002; Keys and Parlin, 1966; Mensink and Katan, 1992;Yu *et al.*, 1995). They reported that saturated fatty acids increased, and polyunsaturated fatty acids decreased TC and LDL-C. When saturated, monounsaturated, and polyunsaturated fatty acids replaced carbohydrates in the diet, they all increased HDL-C and saturated fatty acids had a slightly greater effect than the other two. Since the substitution of saturated fats with carbohydrates of the diet decreased both LDL-C and HDL-C proportionally, it had little effect on the LDL-HDL ratio. Thus substitution would be expected to have less benefit on CHD risk. The monounsaturated and polyunsaturated fatty acids, on the other hand, markedly decreased the LDL-HDL ratio and could decrease CHD risk.

1.3 Liver's Role on The Regulation of Serum Cholesterol at Cellular Level

The plasma LDL-C concentration is determined by the balance between the rate of production and clearance of this lipoprotein from circulation (Brown *et al.*, 1981). The VLDL is synthesized in the liver and serves to transfer triacylglycerol from the liver to peripheral tissue for storage or utilization as a metabolic fuel. As the VLDL triacylglycerol is hydrolyzed, the size of VLDL is reduced. Some of the VLDL remnants, now called Intermediate Density Lipoproteins (IDL), are taken up directly by the LDL-receptor (LDL-r) back into the liver. The rest is converted to LDL and subsequently cleared by the liver though the same mechanism (Dietschy *et al.*, 1993).

Because the liver may play an important role in changing the plasma LDL-C levels, it is suggested that variation of dietary fats may affect LDL-C levels through effects on the liver. However, it is not clear if this involves effects on LDL clearance, cholesterol production, or both.

Cholesterol homeostasis in the liver is regulated by several sterol-sensitive regulatory proteins and receptors, which are primarily associated with the endoplasmic reticulum (ER) and, in some cases, they are associated with plasma or outer nuclear membrane. These include **a)** cholesterol uptake via LDL and other protein receptors (Borchardt and Davis, 1987;Dietschy, 1998); **b)** HMG-CoA reductase (HMG-R), the rate-limiting step in the cholesterol biosynthesis pathway (Orci *et al.*, 1984); **c)** CYP7A1 and CYP27, the initial enzymes in the classic and alternative pathways of bile acid synthesis, respectively (Russell and Setchell, 1992); **d)** acyl-coenzyme A:cholesterol acyltransferase (ACAT) (Chang *et al.*, 1997;Lange *et al.*, 1993) and neutral cholesterol ester hydrolase (CEH), the enzymes that regulate the pool sizes of free and esterifed cholesterol compartments in the liver; **e)** the precursor forms of regulators of transcription (SREPBs) (Horton *et al.*, 2002), and **f)** proteases that modulate some of these factors (SCAP, S1P, S2P) (Edwards *et al.*, 2000). All of these proteins respond to changes in cell cholesterol. In particular, a regulated pool of cholesterol in the ER might serve as a control element in cholesterol homeostasis (Lange *et al.*, 1993).

The existence of a putative ER cholesterol regulatory pool involved in determining the activity of key enzymes just mentioned in cholesterol homeostasis has been postulated for many years (Edwards and Ericsson, 1999). In human fibroblasts, 85% of free cholesterol is in the plasma membrane (Lange *et al.*, 1989) and perhaps 10% is in the

(Lange, 1991). One measure of the ER cholesterol suggests that it contains ~0.5% of the total unesterified cholesterol in the cell (Lange and Steck, 1997). The level of the ER pool is set by a brisk circulation of cholesterol to and from plasma membrane and a small change in plasma membrane cholesterol near the physiologic set point induces large responses in ER cholesterol (Lange *et al.*, 1999). The ER sterol-sensitive proteins then induce cholesterol esterification, decrease its biosynthesis, and suppress transcription factor precursors that activate genes promoting cholesterol accretion. These responses complete a feedback mechanism that maintains the plasma membrane cholesterol pool at the physiological level (Lange *et al.*, 2004).

1.4 The F1B Hamster as a CHD Model

A major challenge in our efforts to completely understand the molecular basis for the initiation and development of CHD is the insidious nature of the disease. A number of animal models have been used including rodents, rabbits, miniature swine and hamsters. Some of these have been more useful than others. The hamster has proved to be a good model to study lipoprotein metabolism since the concentration of serum LDL-cholesterol responds to changes in dietary lipids in a similar manner to that seen in human serum (Spady and Dietschy, 1985; Spady and Dietschy, 1988).

These changes in hamster include similarities to the human: a) cholesterol and bile acid metabolism (Spady and Dietschy, 1983); b) non-high density lipoprotein (LDL) cholesterol and triglyceride response to an atherogenic diet (Spady and Dietschy, 1988); c) development of atherosclerotic lesions similar to those found in early stages of disease (Nistor *et al.*, 1987); d) exclusive hepatic production of apolipoprotein (apo) B-100

(Arbeeny *et al.*, 1992). Thus, the hamster has been used extensively as a model for investigation of the effects of dietary fats on cholesterol regulation and atherogenesis (Parker *et al.* 1995; Kahlon *et al.*, 1996; Nicolosi *et al.* 1998; Margiapane *et al.*, 1999).

The normal Golden Syrian hamster, however, is somewhat resistant to dietary cholesterol-induced atherosclerosis (Nistor *et al.,* 1987; Margiapane *et al.*, 1999. In contrast, a hybrid strain (F1B hamster), has been shown to develop atherosclerotic lesions fairly quickly when fed with low levels of cholesterol (Kowala *et al.*, 1991). Moreover, the F1B hamster develops lesions that are histologically similar to those in humans (McAteer *et al.*, 2003). This has made the F1B hamster an excellent model for study of the effects of dietary fats on the molecular mechanisms involved in atherogenesis.

1.5 Previous Study and Present Focus

This work is a follow up from a previous study in our laboratory carried out by P. de Silva. The previous study was designed to examine the effects of dietary lipids (PUFA and cholesterol) on the regulation of lipoprotein metabolism in the F1B hamster. The animals were fed with 8 different diets rich in n-3 (n-3/n-6=10/1; fish oil diet) or n-6 fatty acids (n-3/n-6=1/10; mix diet) at low (5%, w/w) or high (20%, w/w) fat levels with 0.1 or 0.25% (w/w) cholesterol.

Those results were the first to demonstrate that fish oil caused hyperlipidemia in F1B hamsters. Other key findings from that study were:

a) serum total cholesterol, VLDL- and LDL-cholesterol concentration were significantly higher with lower HDL-cholesterol in hamsters fed with fish oil diets compared to animals fed with mix diets,

b) higher amount of fats in the diets increased plasma lipids in all groups but fish oil groups showed higher serum lipid levels especially in high fat groups,

c) the hepatic cholesterol content was not significantly different between groups,

d) the LDL-receptor mRNA was lower in the animals fed with fish oil diets, and may contradict the well known hypolipidemic and hyperlipidemic effects of dietary n-3 fatty acids and cholesterol; respectively.

In the present study, our goal is to explore cholesterol metabolism in the F1B hamster caused by dietary PUFA and cholesterol at the hepatic cellular level. We looked at the expression levels of several microsomal proteins, including HMG-CoA reductase, ACATA1, CYP7, and transcription factors (SREBPs), involved in the cellular cholesterol homeostasis in hamster liver.

1.6 Feedback Regulation of HMG-CoA Reductase Activity

Feedback regulation of cholesterol biosynthesis was first recognized nearly 50 years ago by Gould and his colleagues (Gould *et al.*, 1953). It takes place primarily in the liver and is exerted mainly on HMG-CoA reductase and plays a critical role in overall cholesterol homeostasis. Many studies have focused on transcription, translation, protein turnover and regulation of catalytic efficiency by phosphorylation/dephosphorylation

regulated at multiple levels (Brown and Goldstein, 1980). However, recent studies on rats showed that a cholesterol-supplemented diet significantly decreased both HMG-CoA reductase activity and protein levels (Chambers and Ness, 1998;Ness *et al.*, 1994), and thus suggested that feedback regulation of hepatic HMG-CoA reductase by dietary cholesterol occurs mainly by decreasing the amount of HMG-CoA protein rather than inactivating the enzyme by phosphorylation.

1.6.2 Different HMG-CoA Reductase Activity Among Various Species

Feeding rats diets containing 2% cholesterol markedly reduced hepatic HMG-CoA reductase activity but had little effect on HMG-CoA reductase mRNA levels (Ness *et al.*, 1991). Rats fed with cholesterol or the bile salt, cholate, showed a marked reduction in the rate of hepatic cholesterol synthesis (by approximately 75% with bile salt, 90% with cholesterol), but only moderate reductions were observed in HMG-CoA reductase mRNA level (40% reduction) (Spady and Cuthbert, 1992). This suggested that dietary cholesterol feedback in rat liver is exerted primarily at the posttranscriptional level.

In contrast to the studies in rats, experiments on cholesterol feedback regulation of hepatic HMG-CoA reductase in both hamsters and mice have revealed an apparent transcriptional regulation. Feeding mice with cholesterol reduced HMG-CoA reductase mRNA level to only 25% of the control in the liver (Rudling, 1992). Similar results are also seen in a hamster model with a decrease of 85% in mRNA level when the diet was supplemented with cholesterol (Gil *et al.*, 1986). These results indicated that dietary cholesterol exerted feedback regulation primarily at transcriptional level in mice and hamsters. Thus the different animal experiments indicate that transcriptional regulation

as a mechanism for feedback regulation of HMG-CoA reductase may be more important in mice and hamsters which are cholesterol-sensitive animals; but it plays a minor role in rats that are relatively dietary resistant to cholesterol.

1.6.3 Regulatory Mechanisms of HMG-CoA Reductase

Recently the transcriptional regulation of HMG-CoA reductase has been shown to involve the binding of sterol regulatory element binding proteins (SREBPs) to sterol regulatory elements (SREs) within the promoter region of the HMG-CoA reductase gene (Brown and Goldstein, 1997;Vallett *et al.*, 1996). This concept of transcriptional control is supported by the observation that treatment with Cholestyramine (a bile acid sequestrant) and Mevinolin (a HMG-CoA reductase inhibitor) increased the amount of HMG-CoA reductase protein in rat liver by elevating the amount of its mRNA. In addition, feeding cholesterol to rats treated with these agents lowered the amount of hepatic HMG-CoA reductase protein by decreasing the level of its mRNA (Liscum *et al.*, 1983). Similar results have been observed using cultured tumor cells (Brown and Goldstein, 1997). The role of SREBPs and their regulation will be discussed later.

1.6.4 Dietary Polyunsaturated Fatty Acids Inhibit HMG-CoA Reductase Activity

The effects of polyunsaturated fatty acids (PUFA) on HMG-CoA reductase have been intensively studied in rats since the 1980's. An early study showed that rat hepatic HMG-CoA reductase activity was inversely correlated with levels of dietary polyunsaturated fatty acids (Ide *et al.*, 1978). Later studies showed that diets supplemented with both n-3 PUFA (fish oil) and n-6 PUFA (safflower seed oil) lower

HMG-CoA reductase activity, but that n-3 fatty acids were much more effective than n-6 fatty acids at reducing the activity (Choi *et al.*, 1989;Mitropoulos *et al.*, 1980). Similar results were also seen when rats were intravenously infused for two weeks with a fat emulsion containing 20% of triacylglycerol in which either n-6 or n-3 fatty acids predominated (al Shurbaji *et al.*, 1991).

Similar studies have also been done with rabbits, that have a much lower hepatic HMG-CoA reductase activity than rats (Shapiro and Rodwell, 1971). The hepatic HMG-CoA reductase activity was further reduced when rabbits were fed for two days with a 1% cholesterol and 5% corn oil mix diet compared to the cholesterol diet without corn oil. This suggests that rabbit HMG-CoA reductase has kinetic properties similar to the rat HMG-CoA reductase (Stange *et al.*, 1981). Another study showed that dietary n-3 fatty acids decreased HMG-CoA reductase activity by 76% compared to normal rabbit chow, and additional cholesterol (1%) further reduced the activity to 10% of the control (Field *et al.*, 1987). The effects of dietary unsaturated fatty acids on hepatic HMG-CoA reductase found in mice are similar to those seen in rats and rabbits. Compared to n-6 PUFA, supplementation for one week with dietary n-3 fatty acids further reduced the specific activity of the HMG-CoA reductase. These differences were due, at least in part, to differences in the amount of HMG-CoA reductase protein (El Sohemy and Archer, 1999). These results have been confirmed in a recent long-term feeding study (Du *et al.*, 2003).

A long-term feeding experiment on mice indicated that the hypocholesterolemic activity of dietary polyunsaturated fatty acids is exerted by n-3 fatty acid-rich oils by

suppressing hepatic HMG-CoA reductase activity compared with animal fats and high-linoleic (n-6) oil (Du *et al.*, 2003).

In conclusion, dietary n-3 fatty acids were a major factor in the dietary unsaturated fatty acid mediated regulation of cholesterol biosynthesis and they exerted their effect by suppressing hepatic HMG-CoA reductase activity. Additional dietary cholesterol further reduced the HMG-CoA reductase activity.

1.7 Cholesterol 7 alpha-hydroxylase (CYP7A1 hydroxylase)

Bile acid synthesis from cholesterol is a major pathway for elimination of cholesterol from the body, occurring either via the classic (also called "natural") or alternative (also called "acid") bile acid synthesis pathways (Vlahcevic *et al.*, 1999). Bile acids represent the terminal end products of cholesterol catabolism, and their synthesis takes place exclusively in the liver (Vlahcevic *et al.*, 1999). The pathway of bile acid synthesis in the enterohepatic circulation is controlled by a microsomal enzyme known as cholesterol-7α-hydroxylase (CYP7A1). The activity of CYP7A1 is regulated by a variety of nutritional and hormonal factors involving both feedback and feedforward mechanisms. The regulatory control appears to be exerted mainly at the level of gene transcription, because CYP7A1 activity is highly correlated with its mRNA level.

1.7.1 Regulation of CYP7A1 Activity

Many experiments on rodents showed that CYP7A1 gene expression is suppressed by bile acids and stimulated by cholesterol. The stimulation of CYP7A1 gene expression by cholesterol is mediated through liver X receptor (LXRα), an oxysterol-activated

nuclear receptor that binds to the CYP7A1 gene promoter as a heterodimer with another nuclear receptor known as retinoid X receptor (RXR) (Lehmann *et al.*, 1997;Peet *et al.*, 1998). Different species vary widely in their response to dietary cholesterol. The rat responds to cholesterol feeding with induction of CYP7A1 and bile acid synthesis, is relatively resistant to development of hypercholesterolemia. In contrast, increased dietary cholesterol fails to induce CYP7A1 in rabbits (Xu *et al.*, 1996), green monkey (Rudel *et al.*, 1994), and hamsters (Horton *et al.*, 1995). In these species, excess cholesterol results in the development of hypercholesterolemia and atherosclerosis. The ability of most humans to respond to excess dietary cholesterol is probably more like the hamster. In humans, the active LXR site is absent in the CYP7A1 promoter. This region of the human CYP7A1 promoter contains a hepatocyte nuclear factor-1 (HNF1) binding site (Chen *et al.*, 1999;Molowa *et al.*, 1992). Differences in LXRα activation sites in the CYP7A1 promoter may explain the species-specific difference in response to dietary cholesterol.

The inhibition of CYP7A1 gene expression by bile acids is mediated through an indirect mechanism. Previous studies identified the nuclear receptor FXRα as the major hepatic bile acid sensor that governs bile acid synthesis and transport (Clifford *et al.*, 1976;Mortara *et al.*, 1976;Wang *et al.*, 1999). Bile acids are potent ligands of FXRα, which induces the expression of SHP (small heterodimer partner). Elevated levels of SHP in turn lead to transcriptional repression of the CYP7A1 gene, by inhibiting the activity of nuclear receptor LRH-1 on the CYP7A1 promoter (Goodwin *et al.*, 2000;Lu *et al.*, 2000). SHP is an atypical nuclear receptor lacking a DNA-binding domain (Seol *et al.*, 1996). It contains an N-terminal receptor dimerization domain, which mediates its

recruitment to promoter via interaction with various nuclear receptors. SHP is expressed at low levels in the liver (Goodwin *et al.*, 2000;Lu *et al.*, 2000).

1.7.2 Effects of PUFA on Bile Acid Synthesis

Recently, the liver X receptors (LXRα and LXRβ) were identified as targets for fatty acid regulation (Ou *et al.*, 2001). Unsaturated fatty acids antagonize oxysterol activation by LXRα in Hek 293 and hepatoma cell lines by interfering with oxysterol binding. Although such studies suggest that changes in hepatic PUFA levels might affect bile acid synthesis *in vivo,* feeding studies with mice have yet to support this view. Interestingly, hepatic 7α-hydroxylase (CYP7A1) activity and its mRNA levels are not suppressed in mice fed diets supplemented with unsaturated fatty acids (Cheema *et al.*, 1997;Cheema and Agellon, 1999).

1.8 Acyl-CoA:cholesterol acyltransferase (ACAT)

Acyl-CoA:cholesterol acyltransferase (ACAT) is an integral enzyme present in the rough ER that catalyzes the esterification of cholesterol from free cholesterol and fatty acyl coenzyme A. This mechanism is apparently in response to an increased ER cholesterol pool and believed to be activated as a means to remove excess cholesterol, and the esters are deposited in the cytosolic lipid droplets (Simons and Ikonen, 2000). Under pathological conditions, cholesterol esters produced by the ACAT reaction accumulate as lipid droplets in macrophages and eventually cause foam cell formation in the early stage in the formation of atherosclerotic plaques (Chang *et al.*, 2006). Due to the technical difficulties of directly tracing the cholesterol movement in the cell, the level

of cholesterol esterification has become the standard method for monitoring the cholesterol transport to the ER and is assumed to reflect the ER cholesterol pool (Debry *et al.*, 1997; Lange and Steck, 1997).

Two isoforms of ACAT (ACAT-1, ACAT-2) were found in several species including mice (Cases *et al.*, 1998), humans (Oelkers *et al.*, 1998) and other primates. ACAT-1 is ubiquitously expressed with its active site oriented toward the cytosol, and mainly served to protect cell membranes from the toxicity of excess free cholesterol. ACAT-2, however, is expressed primarily in the liver and intestine with the active site faced towards the lumen of the ER, suggesting that ACAT-2 may play an important role in the synthesis and secretion of hepatic lipoproteins, and the cholesterol absorption from small intestine (Carr *et al.*, 1995). While the structure and general function of ACAT are well defined, less is known of the factors that regulate ACAT activity.

1.8.1 Regulation of ACATA1 Activity

ACATA1 appears to be regulated mainly by post-translational mechanisms (Chang *et al.*, 1997). Cholesterol esterification is elevated in cholesterol-loaded cells without changes in ACATA1 mRNA (Matsuda *et al.*, 1996; Rea *et al.*, 1996; Wang *et al.*, 1996) or protein expression levels (Chang *et al.*, 1994; Wang *et al.*, 1996; Yu *et al.*, 1999). The *in vitro* studies using purified ACATA1 support the hypothesis that ACATA1 is allosterically activated by binding to cholesterol or oxysterol (Chang *et al.*, 1998). ACAT may also be regulated at the transcriptional level. The ACATA1 mRNA level increased after feeding a cholesterol-rich diet to mice (Uelmen *et al.*, 1995) or rabbit (Pape *et al.*, 1995). The significant increase of mRNA was also found in human

macrophages during the differentiation of monocytes into macrophages (Wang *et al.*, 1996).

1.8.2 Effect of PUFA on ACATA1 Activity

Early in vivo studies have shown that, compared with saturated fat diets, diets high in unsaturated fatty acids increase liver ACAT activity (Field *et al.*, 1987;Spector *et al.*, 1980). In vitro studies suggested that unsaturated fatty acids also directly induce cellular ACAT activity (Rumsey *et al.*, 1995). However recent in vitro studies showed that different unsaturated fatty acids induce ACATA1 mRNA levels in cell specific (HepG2) manner; this does not appear to correlate with the ACAT activity (Seo *et al.*, 2001). Interestingly hepatic ACAT activity was lower in hamsters fed dietary fats containing acyl groups with 18:1c and 18:2c fatty acids compared with those fed diets containing 16:0 (Bhatty *et al.*, 2001).

1.9 Sterol Regulatory Element Binding Proteins (SREBPs)

With the recent discovery of the sterol regulatory elements (SREs) in the promoter region of genes, a family of transcription factors, called sterol regulatory element binding proteins (SREBPs), has been suggested to regulate more than 30 genes involved in the cellular homeostasis of cholesterol and fatty acids (Brown and Goldstein, 1997;Edwards *et al.*, 2000;Horton and Shimomura, 1999;Sakakura *et al.*, 2001). In cholesterol metabolism, SREBPs activate target genes encoding HMG-CoA synthase (Smith *et al.*, 1988), HMG-CoA reductase (Vallett *et al.*, 1996), farnesyl diphosphate synthase (Ericsson *et al.*, 1996) and squalene synthase (Guan *et al.*, 1997), and LDL-receptor

which mediates the cellular uptake of cholesterol from plasma (Brown and Goldstein, 1986).

SREBPs belong to the basic helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors. They are first synthesized as inactive precursors, bound to rough endoplasmic reticulum (ER), containing approximately 1150 amino acids organized into three domains: (i) an NH_2-terminal of about 500 amino acids containing bHLH-Zip region for DNA binding; (ii) a middle hydrophobic region of about 80 amino acids containing two hydrophobic transmembrane-spanning segments interrupted by a short loop of about 30 amino acids that projects into the lumen of ER; and (iii) the COOH-terminal regulatory domain of about 590 amino acids. Both NH_2-and COOH- terminals project into the cytosol of cells (Brown and Goldstein, 1997).

To date, three SREBP isoforms have been identified in mammalian cells. SREBP-1a and SREBP-1c are derived from a single gene by use of alternative promoters and splicing, resulting in different forms of exon-1. The SREBP-2 is encoded by a separate gene. In growing cultured cells, the SREBP-1a and SREBP-2 are predominant; but in most animal tissues, the SREBP-1c and SREBP-2 are predominant. SREBP-1 has been shown to preferentially activate genes required for fatty acid synthesis; in contrast, SREBP-2 preferentially activates genes involved in cholesterol homeostasis (Shimomura *et al.*, 1997).

1.9.1 Regulation of SREBP by Cholesterol

In order to enter the nucleus and activate target genes, the N-terminal bHLH-Zip domain, often referred to as the nuclear form of SREBPs (nSREBPs) must be released from the ER membrane by a two-step proteolysis. Initially SREBP precursor (pSREBP) and SCAP are both inserted into the membrane of rough ER with a protein-protein interaction which is required for SCAP to control the processing of SREBP (Sakai *et al.*, 1997; Sakai *et al.*, 1998). When cells need cholesterol, SCAP senses this need through its membranous sterol-sensing domain and escorts the SREBP from the ER to the Golgi apparatus where site-1 (S1P) and site-2 (S2P) proteases await. In the Golgi, a membrane-bound serine protease, S1P, cleaves the SREBP in the luminal loop dividing the precursor in half. Then S2P, a membrane-bound zinc metalloproteinase, cleaves SREBP in the first transmembrane domain releasing the N-terminal domain, nSREBP, from the ER membrane. The nSREBP then enters the nucleus and binds to SRE in the promoter regions of target genes, including HMG-CoA reductase and LRL-receptor, for transcriptional activation. When cholesterol is abundant in the cells, the SREBP/SCAP complex is retained in the rough ER and no cleavage occurs. Therefore the nSREBP can not enter the nucleus to activate SER-related genes. The mechanism by which SCAP senses the cellular sterol level and escorts the SREBP/SCAP complex from ER to Golgi is still unknown (Horton *et al.*, 2002).

Recently other integral-membrane proteins, insulin-induced gene-1 (Insig-1) and Insig-2 have been found in the ER, which may bind to the SCAP and cause the ER retention of SCAP/SREBP complex (Yabe *et al.*, 2002; Yang *et al.*, 2002). In Drosophila cells, sterols block transport of the mammalian SCAP/SREBP-2 complex only when

Insig-1 or Insig-2 is co-expressed (Dobrosotskaya *et al.*, 2003). The addition of cholesterol to the ER membranes causes a conformational change in the cytoplasmic loop of SCAP, and the amount of cholesterol required for this conformational change is decreased in the presence of Insig proteins, therefore facilitating the retention of SCAP/SREBP complex in the ER (Adams *et al.*, 2003). In addition, Insig-1 has been shown to enhance the degradation of HMG-CoA reductase by binding to its sterol-sensitive domain when cholesterol levels are high (Sever *et al.*, 2003), suggesting a dual role for Insigs in the SCAP/SREBP pathway and the regulation of cholesterol metabolism.

1.9.2 Effects of Unsaturated Fatty Acids on SREBPs

Dietary polyunsaturated fatty acids have been well established as negative regulators of hepatic lipogenesis. Thus, several laboratories have investigated whether the unsaturated fatty acids have feedback effects of SREBPs. Briefly, these reports all indicated that PUFA suppress the expression of SREBPs, but the mechanism is different in cell culture and in animals. Xu et al. (Xu *et al.*, 1999) found that dietary (n-3) and (n-6) PUFA, but not saturated or (n-9) unsaturated fatty acids unequally reduce the hepatic abundance of SREBP-1 mRNA and both the precursor and nuclear form of SREBP-1. This inhibition is paralleled by significant reduction in the transcription of hepatic fatty acid synthesis. A later study showed that PUFA decrease the hepatic SREBP-1 by accelerating the rate of mRNA decay (Xu *et al.*, 2001). Kim et al. (Kim *et al.*, 1999) found that long-term feeding of mice with a fish oil diet enriched in n-3 PUFA decreased nuclear form of both SREBP-1 and -2 in the liver. The mRNA of SREBP-1 was reduced

significantly, but SREBP-2 was only partially reduced. Recently, Hannah et al. (Hannah *et al.*, 2001) suggested that not only the unsaturated fatty acids reduce the nSREBP-1 in HEK-293 cells by suppressing the proteolytic release of nSREBP-1, but they found that 16:1(n-9) and 18:1(n-9) were as effective as n-3 and n-6 PUFA at suppressing the processing of SREBP-1. Therefore fatty acids may exert their effects on SREBPs differently in animals and cell lines.

1.10 Hypothesis and objectives

Based on the current understanding of the regulation of cholesterol metabolism by dietary fatty acids and cholesterol we proposed that the hyperlipidemic effects of dietary fat observed in the F1B hamster reflects abnormalities in the regulatory mechanisms in the liver of this atherosclerosis prone animal model, including the SREBP mediated control of cholesterol biosynthesis.

The objective of this study was to investigate the biochemical regulation of cholesterol metabolism in the liver of F1B hamsters caused by different dietary PUFA and cholesterol. We looked at the expression of several microsomal enzymes that play key roles in cholesterol metabolism in liver including, HMG-CoA reductase, CYP7, ACAT, a corresponding transcription factor, SREBP-2,. We also examined the lipid composition in the microsomal membrane in the animals fed different diets.

Chapter 2. Methodology

2.1 Animals and Diets.

The F1B hamsters (male, 7 weeks of age) were purchased from Bio Breeder Inc. (Water Town, MA) and fed a chow diet for a one week equilibration period. This was reported to be sufficient time to allow the animals to recover from shipping and acclimatize to new housing (Landi *et al.,* 1982; Dymsza *et al.*, 1963). This also allowed the animals to become adjusted to the 12h light/ 12h dark conditions as the plasma lipid response in the F1B hamster has been reported to be sensitive to both photoperiod and caging (Smith *et al.*, 2001). The animals were separated into 8 groups and fed with one of the experimental diets. The contents of the diet included fat free semi-purified diet (ICN Biomedical INC., Cleveland, OH, catalog # 960241) and either fish oil (menhaden oil) or a mixture of lard and safflower oil in 1.5:1 ratio (mix diet). Lard and safflower oils were obtained from a local supermarket. The components of the diets are shown in Table 2.1. Diets were prepared with two levels of fat, 5% (w/w) (low fat) and 20% (w/w) (high fat). Due to the presence of small amounts of cholesterol in the fish oil, a similar amount of cholesterol (98% pure, Sigma-Aldrich, St. Louis, MO) was added to the MIX diets to make the cholesterol levels similar to the fish oil diets. In order to elevate the cholesterol level to a concentration of 0.25% (w/w) in both fish oil and MIX diets, additional cholesterol was added to both diets. The fatty acid composition of the diets is given in Table 2.2.

The animals were housed in a controlled environment with a 12hr (lights on from 07.00 to 19.00 hours) light/dark cycle and were given free access to water and diets. The temperature was maintained at 21°C, with humidity kept at 35±5 %. After two weeks on the specific diets, some of the animals started to lose weight, thus for this pilot project we

Table 2.1 Components of the Experimental Diets at Low (5%) and High (20%) Fat Level. (%, w/w)

	Low Fat Diet[a]				High Fat Diet[b]			
	Fish Oil Diet		Mix Diet[c]		Fish Oil Diet		Mix Diet[c]	
Components[d]	Low Chol	High Chol	Low Chol	High Chol	Low Chol	High Chol	Low Chol	High Chol
Sucrose	50	50	50	50	30.5	30.5	30.5	30.5
Casein	20	20	20	20	20	20	20	20
Maize starch	14.6	14.6	14.6	14.6	19	19	19	19
DL-Methionine	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Mineral mix[e]	4	4	4	4	4	4	4	4
Vitamin mix[e]	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Fiber[f]	5	5	5	5	5	5	5	5
Fat	5	5	5	5	20	20	20	20
Cholesterol	0.1	0.25	0.1	0.25	0.1	0.25	0.1	0.25

chol, cholesterol

[a]Semi-purified diet designed for 5% fat level.

[b]Semi-purified diet designed for 20% fat level.

[c]Mix diet, semi-purified diet (MP Biomedicals # 960241) supplemented with lard and safflower-seed oil.

[d]Components were from MP Biomedicals, Solon, OH, USA.

[e]Supplied in adequate amounts to meet requirement (National Research Council, 1995). AIN-76 Mineral mix ;Vitamin mix (MP Biomedicals # 904654).

[f]Cellulose was supplied as Alphacel non-nutritive bulk (MP Biomedicals, Solon, OH, USA).

Table 2.2 Fatty Acid Composition of the Diets (%, w/w).

Lipids were extracted from the diets and the fatty acid composition of the diets was determined by gas-liquid chromatography (GLC) using a Hewlett Packard 5890 instrument equipped with a flame ionization detector (FID). Methyl esters were prepared as described in Keough *et al.* (1979).

Fatty acids	Fish oil	Mix
14:0	9.6	1.0
16:0	19.3	19.3
16:1 n7	13.1	2.0
18:0	3.8	10.0
18:1 n9	13.8	31.0
18:2 n6	2.7	34.0
18:3 n3	4.5	3.0
18:4 n3	3.4	ND
20:1 n9	1.6	ND
20:4 n6	1.0	ND
20:5 n3	12.9	ND
22:5 n3	2.4	ND
22:6 n3	12.0	ND
ΣSFA	32.0	30.0
ΣMUFA	28.0	32.0
ΣPUFA	38.0	37.0
Σn-3 PUFA	35.0	3.0
Σn-6 PUFA	3.0	34.0

ΣSFA, sum of saturated fatty acids.
ΣMUFA, sum of monounsaturated fatty acids.
ΣPUFA, sum of polyunsaturated fatty acids.
ND, not detected.

decided to terminate the experiments at two weeks of feeding. All animals were sacrificed after 14 hours fasting (9:00 am). The livers were removed and quick frozen in the liquid nitrogen and stored at –70°C until further use.

2.2 Preparation of Microsomes from Hamster Livers.

Approximately 300mg of hamster liver was homogenized with 3ml ice-cold buffer I (50mM KCl, 1mM EDTA, 100mM K_2HPO_4 (pH 7.4), 50mM KF, 5mM Dithiothreitol (DTT), 300mM sucrose) and centrifuged at 9,000rpm (9,800g) at 4°C for 20 minutes (Beckman JA-20 rotor). The fat on the top of the supernatant was removed. The supernatant was then centrifuged at 35,000rpm (150,000g) at 4°C for 70 minutes (Beckman SW 55 Ti rotor). The microsomal pellets were resuspended with 500µl of buffer II (50mM KCl, 1mM EDTA, 100mM K_2HPO_4 (pH 7.4), 50mM KF and 50mM DTT). All procedures were performed at 4°C.

2.3 HMG-CoA Reductase Activity Assay.

The HMG-CoA reductase activity was determined to be linear from 0-400ug of protein. The hamster liver microsomes with 0.3mg of protein were first mixed with 0.5 mM DTT and 2X CYP7 assay buffer (200mM K_2HPO_4, pH 7.4, 2mM EDTA-K_2, 100mM KF) to bring the volume to 100µl and preheated to 37°C for 5 minutes. Incubations were carried out by adding 50 μl of a substrate mixture containing 0.1 μCi of 3-Hydroxy-3-methyl [3-^{14}C] glutaryl-coenzyme A (50-62mCi/mmol), 50 nmol of DL-3-Hydroxy-3-methylglutaryl coenzyme A, 0.4 μmol NADP, 0.6U of Glucose-6-Phosphate Dehydrogenase, 5.3 μmol of β-D-Glucose 6-phosphate, 0.09M EDTA-K_2, 0.15M

K_2HPO_4, pH 7.4, 0.068M KF, and incubated in a 37°C water bath. After 25 minutes, the reaction was stopped by adding 20 μl of 5N HCl and kept at 37°C for another 30 minutes to assure lactonization of the mevalonate (Panini *et al.*, 1984). The denatured proteins are then removed by centrifugation (12,000g /5min). 150μl of supernatant was transferedto a micro tube containing 10μl of 0.77M mevalonic acid lactone. The supernatants were spotted at one inch from the bottom on Whatman glass TLC plates (K6 Cilica Gel). The plates were developed in acetone-benzene (1:1) until the solvent front was within 1 cm of the top edge of the plate. After the lipids were visualized using iodine vapor, the C^{14}-mevalonic acid lactone was identified by comparision with a mevalonic acid lactone standard. After complete removal of iodine the mevalonic acid lactone band was scraped from the plate and counted in a liquid scintillation counter (LKB Model 1214 Rackbeta).

2.4 CYP-7 Activity Assay

Cpy-7 enzyme activity in purified microsomes was assayed using a modification of previously described method using isotope incorporation into 7α-hydroxy cholesterol (Martin *et al.*, 1993). In the assay, [^{14}C] cholesterol (53.10mCi/mmol) (0.04mCi/ml) encapsulated in 2-hydroxypropyl-β-cyclodextrin (Sigma) was used as a substrate. In a total volume of 250ul, each assay tube contained 100mM K_2PO_4, PH 7.4, 1mM EDTA-K_2, 50mM KF, 1mM NADPH, [^{14}C] cholesterol (0.0612 uCi), 4.5 ug cholesterol, 2.205mg cyclodextrin, 300ug microsome protein. Before adding NADPH, the assay tubes containing all components were preincubated on ice. The tubes were prewarmed in a 37°C water bath for 3min, and the assay started with addition of NADPH and incubated at 37°C for 15min. The reaction was stopped with 20ul of 5M NaOH. The final product,

7α-hydroxycholesterol, was extracted with ethyl acetate and separated from the reaction mixture by thin-layer chromatography using an ethyl acetate-toluene (3:2 v/v) solvent system. The product spot was visualized by iodine vapor and scraped from the plate. The amount of radioactivity in the spots corresponding to [^{14}C] 7α-hydroxycholesterol was counted using scintillation counter (LKB Model 1214 Rackbeta). Calculation of enzyme activity took into account the dilution (C^{14}-Chol/Chol=6.22dpm/pmol) of the exogenous labeled cholesterol by the endogenous cholesterol in the microsome.

2.5 ACAT Activity Assay

ACAT activity in microsome fractions was determined using the modified isotope method as described previously (Stahlberg *et al.*, 1989). Each ACAT assay reaction mixture contains 0.1 mg microsomal protein, 1.0 mg bovine serum albumin (Sigma), and cholesterol emulsion with 23 ug (59 nmol) cholesterol (Sigma) and 230ug phosphatidylcholine, 2mM DTT, 0.1M K_2HPO_4 in a total volume of 50ul. The mixture was incubated in a 37°C water bath for 30 min. The reaction was started with additional C^{14}-oleoyl CoA plus 0.2mg cold oleoyl CoA in a 2 ul volume into the mixture and incubated at 37°C for 20 min. After terminating the reaction with additional 200ul of chloroform/methanol (2:1) mixture, the final product, cholesteryl-C^{14}-oleate, was extracted with chloroform/methanol mixture (2:1). The C^{14}-cholesterol ester was separated by thin-layer chromatography (peteroleum ether: diethyl ether: acetic acid= 90:10:1). After the lipids were visualized using iodine vapor, and the cholesteryl ester was identified by comparision with a cholesteryl ester standard. After complete removal of iodine the cholesteryl-ester bands were scraped from the plate and counted.

2.6 RT-PCR Assay for HMG-CoA Reductase mRNA Level.

The total RNA was isolated from hamster liver by using FastRNA kit, and the FastPrep FP120 instrument, following the kit's protocol (Qbiogene, Carlsbad, CA). The purity of the RNA was checked by running the samples on a 1% agarose gel in borate buffer. The mRNA level of HMG-CoA reductase was determined by reverse transcription and amplified by PCR. The complementary DNA was synthesized from the liver RNA (2μg) using Superscript™ reverse transcriptase (from Invitrogen) and was used as the template for PCR amplification. The primers for HMG-CoA reductase were purchased from Sigma-Aldrich and were as follows: sense 5'-CCTCTCCACAAAGCTTCCAG-3', and antisense 5'-CAGAATCACAAGCACGAGGA-3' (accession#: M12705). The HMG-CoA reductase mRNA levels were normalized to β-actin mRNA levels. The primers used for β-actin were: sense 5'-CATCGTACTCCTGCTTGCTG-3', and antisense 5'-GCTACAGCTTCAC-CACCACA-3'. After PCR amplification, the products were resolved on a 1.5% agarose gel. The bands were quantified using the ChemiImager™ 4400 gel documentation system (Alpha Inotech Corporation, San Leandro, CA).

2.7 The Western Blot Assay for SREBP-2.

The frozen liver samples were first homogenized in SDS sample buffer (62.5mM Tris-base, 2% SDS, 10% glycerol and 50mM DTT), and amount of protein in the liver lysate was quantified by Lowry assay after TCA precipitation. The samples (60μg) were boiled and loaded on a 10% SDS-polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane (0.2um) (Bio-Rad Laboratories, Inc., Hercules,

CA). The primary antibody, goat polyclonal IgG, SREBP-2 (N-19), and the secondary antibody, bovine anti-goat IgG-HRP, were used to detect the N-terminal of SREBP-2. SREBP-2 (1C6), the mouse monoclonal IgGI, and bovine anti-mouse IgG-HRP were used to detect the C-terminal of SREBP-2. All antibodies were purchased from Santa Cruz Biology Inc (Santa Cruz, CA). Protein mobilities were compared with molecular weight standards (New England BioLab Inc., Ipswich, MA). The protein bands were then visualized through the chemiluminescent method (Bio-Rad Laboratories, Hercules, CA)

2.8 Quantitation of Microsomal Cholesterol

The microsomal free cholesterol concentration was determined by a previous method (Rudel and Morris, 1973). Briefly, an appropriate amount of microsome (50ul, ~250-400ug protein) was extracted with 220 ul chloroform: methanol mixture (1:1, v/v) and back washed with 50 ul of water. After the extract was centrifuged at 12,000g for 2min, the top layer water was taken off, and the bottom layer solvent was dried with nitrogen.

The assay started with adding 900 ul o-phthalaldehyde (0.5mg/ml acetic acid) and 450 ul concentrated H_2SO_4, to the dried cholesterol and mixed well. After 30min, the absorbance was measure at 550nm. A series of cholesterol standard (0-50nmol) was used and produced a linear relationship between cholesterol concentration and the absorbance at 550nm.

2.9 Quantitation of Microsomal Phospholipid and Fatty Acids

The method used for fatty acid extraction and analysis was previously described (Keough and Davis, 1979). Briefly, the microsomal lipids were extracted as described for cholesterol and transmethylated with 6 % H_2SO_4 in methanol. After heating samples at 90°C for 5 hours, the fatty acid methyl esters (FAME) were extracted twice with hexane and wash with water. The extracts were dried with nitrogen and ~10ul CS_2 was added before injection onto a supelcowax 10 capillary column in an H/P 5890 gas chromatograph. The column program was set at 170°C for 2 minutes and heating from 172°C to 220°C at a rate of 2°C per minute for 27 minutes. The FAME were detected using a FID and identified by retention time.

The method used for phospholipids analysis was previously described (Parrish, 1999). Briefly, an appropriate amount of lipid extract (~7ul) was spotted on chromarods (quartz rod coated with a thin layer of silica) and developed in acetone 5 min for focusing the sample spots, dried and developed for another 7 min to separate the phospholipids from neutrolipids, which were removed by burning in a hydrogen flame. After condition for 2 min, the remaining phospholipids were focused and developed in a polar solvent mixture (chloroform: methanol: water = 70: 35: 3.5). The rods were scanned in an Iatroscan Mark V analyzer (Iatron Laboratories, Tokyo, Japan) and the three chromatograms were combined using T-data scan software (RSS, Bemis, TN). The signal was quantified using lipid standards (Sigma Chemical Company, St. Louis, MO).

2.10 Statistical Analysis

Data are presented as group means ± SD, where n=6 unless otherwise noted. Differences due to the dietary fat amount, fat types, cholesterol, and interactions were determined by three-way analysis of variance (ANOVA) via using the software, Sigmaplot-10. Linear regression was used to assess significant correlations between variables. A *P*-value of less than 0.05 was considered significant.

Chapter 3. Results

3.1 Food Consumption and Hamster Body Weight

Food consumption measurements indicated no change in food intake in hamsters fed the various diets (de Silva, 2003). There was no significant effect of dietary fat levels or diet types on body weight gain in the two week period. However there was a significant increase in liver weight in hamsters fed the high fat diet compared to hamsters fed the low fat diet irrespective of the diet types.

This work forms part of a pilot study and two weeks was selected as an initial feeding period. It has already been reported that the plasma lipid concentrations dramatically increased in the fish-oil-fed hamsters after two weeks of feeding. Moreover, red blood cells from fish oil diet-fed hamsters showed increased levels of EPA and DHA at the expense of linoleic acid (18:2 n-6) and arachidonic acid (20:4 n-6) compared to MIX diet-fed hamsters. Thus, the length of the experimental feeding period was sufficient to induce significant changes in the fatty acid composition of the tissues and these changes reflected dietary lipid intake (de Silva, 2003).

3.2 HMG-CoA Reductase Activity

Figure 3.1 shows the activity of HMG-CoA reductase in the livers of hamsters fed varying amounts of either a fish oil diet or a MIX diet, in the presence of 0.1% or 0.25% cholesterol for two weeks. The additional cholesterol supplement had a significant effect ($P<0.05$) on suppressing the hepatic activity in F1B hamsters, regardless of the types or amount of the fat in the diets. However the difference in activity in the microsomes of animals fed different diets was very modest, especially in those fed high fat fish oil diets.

Figure 3.1 The Dietary Effects on Hepatic HMG-CoA Reductase Activity in F1B Hamsters.

The activity of HMG-CoA reductase was affected significantly by dietary cholesterol treatment only. Hamsters were fed either a fish oil supplemented diet or a control diet supplemented with a mixture of lard and safflower-seed oil (MIX diet), at low fat (5%, w/w) or high fat (20%) levels, in the presence of 0.1% (Grey bars) or 0.25 % (Black bars) (w/w) cholesterol respectively for two weeks. Mean values are shown, with standard deviations indicated by vertical bars (n=6 in low fat diets, n=5 in high fat diets). Differences between groups were evaluated using 3 way ANOVA analysis with Holm-Sidak method.

Three-way analysis of variance (ANOVA) on HMG-CoA reductase Activity

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	138.2	138.2	2.931	0.096
Type of fat (B)	1	3.285	3.285	0.0697	0.793
-/+ Cholesterol (C)	1	201.933	201.933	4.282	**0.046**
A X B	1	72.409	72.409	1.536	0.224
A X C	1	122.929	122.929	2.607	0.116
B X C	1	25.494	25.494	0.541	0.467
A X B X C	1	33.663	33.663	0.714	0.404
Residual	34	1603.239	47.154		
Total	41	2253.123	54.954		

DF – Degrees of Freedom

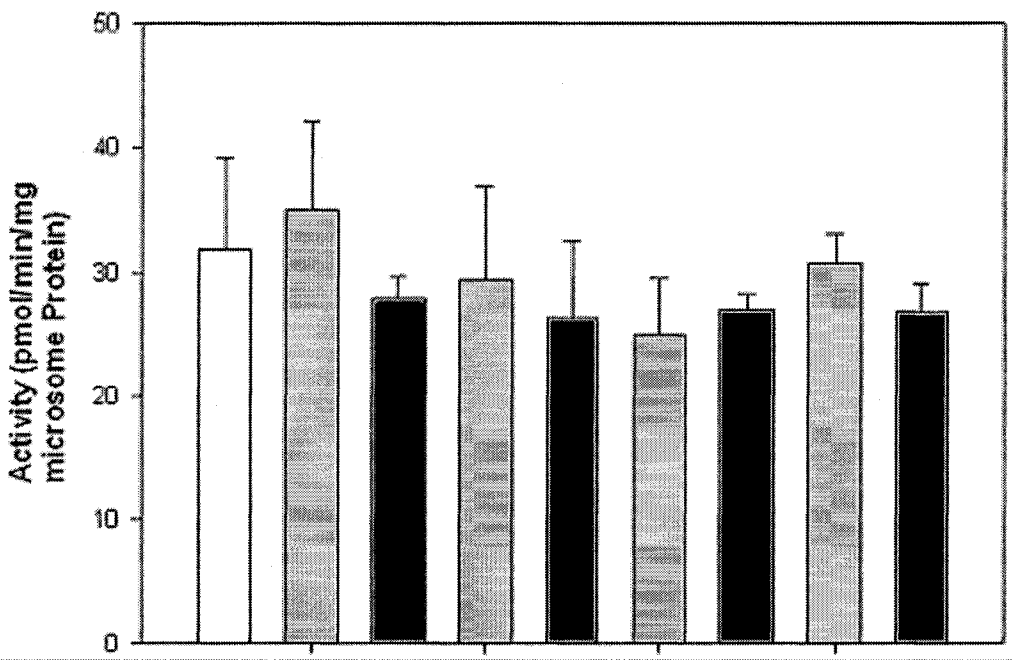
SS – Sum of the Squares

MS – Mean Squares

F – F Distribution

P – p value

Hepatic HMG-CoA Reductase Activity of F1B Hamster



Fat level	—	Low fat (5%)				High fat (20%)			
Dietary type	control	FO		MIX		FO		MIX	
Cholesterol	—	—	+	—	+	—	+	—	+

3.3 mRNA Level of HMG-CoA Reductase

Figure 3.2 shows the mRNA levels of HMG-CoA reductase in the livers of F1B hamsters fed the designed diets for two weeks. Although the mRNA slightly increased when dietary cholesterol is added, the difference was not statistically significant; thus, the dietary interaction had no effect on the mRNA expression.

3.4 CYP7 Activity

Figure 3.3 shows the activity of hepatic CYP7 in F1B hamsters fed various diets for two weeks. The activity was significantly reduced ($P<0.05$) when the proportion of the dietary fat was increased from 5% to 20%, but the types of the polyunsaturated fatty acids, n-3 and n-6, in the diet, and the additional dietary cholesterol had no effect on the CYP7 activity.

3.5 Dietary Effects on Hepatic ACAT Activity

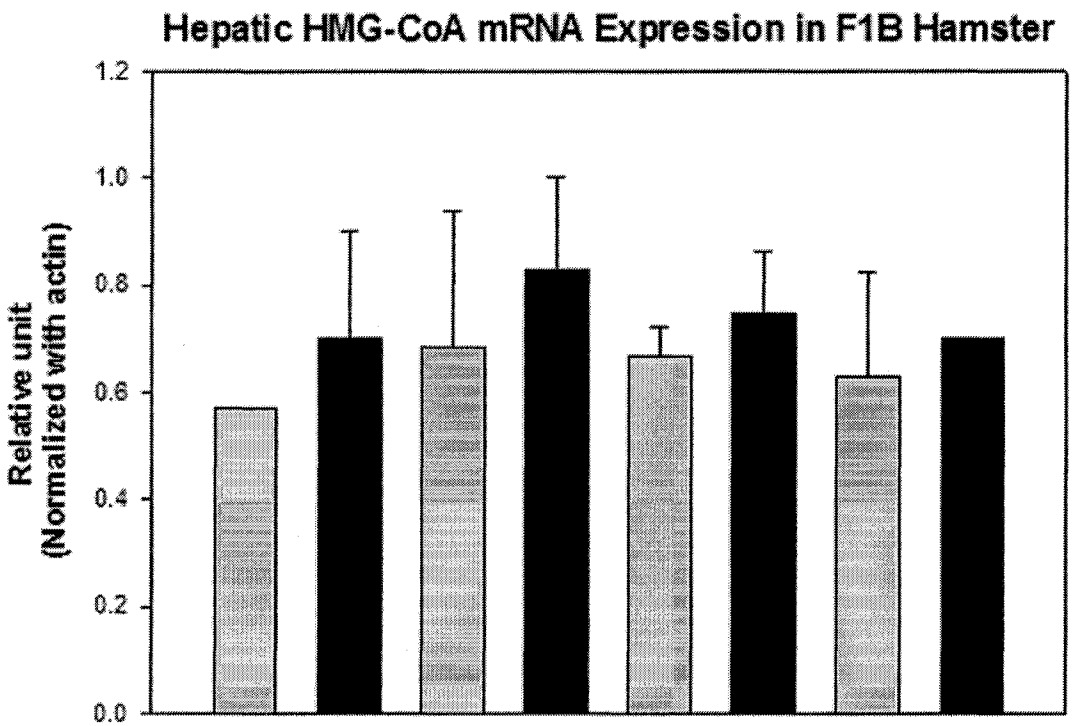
The effects of diet on the levels of Acyl Coenzyme A: Cholesterol Acyltransferase (ACAT) activity in the microsome from F1B hamsters is shown in Figure 3.4. The results revealed that addition of cholesterol to the diet of the animals had a significant effect on ACAT activity ($P<0.05$). The effect was most pronounced in the animals fed a high fat diet where increasing the dietary cholesterol from 0.1% to 0.25% resulted in a marked increase in ACAT activity by 2.5 fold and 1.7 fold for hamsters fed the fish oil and MIX diets respectively. This is reflected in the results from low and high fat diets that shows a significant interaction of dietary fat level and cholesterol content ($P<0.001$). Interestingly the effect of dietary cholesterol on ACAT activity in hamsters fed high

Figure 3.2 The Dietary Effects on The Expression of Hepatic HMG-CoA mRNA in F1B Hamsters.

Diet composition had no significant impact on the mRNA expression of hepatic HMG-CoA reductase in F1B hamsters. The animals were fed either a fish oil supplemented diet or a control diet supplemented with a mixture of lard and MIX diet, at low fat (5%, w/w) or high fat (20%) levels, in the presence of 0.1% (Grey bars) or 0.25 % (w/w) (Black bars) cholesterol for two weeks. Total hepatic RNA was extracted, then reverse transcribed and then the cDNA templates of the reductase and ß-actin were amplified. The amount of amplified templates were quantified and the abundance of HMG-CoA reductase mRNA was expressed relative to ß-actin mRNA. Mean values are shown, with standard deviations indicated by vertical bars (n=3). The lack of the error bars is due to insufficient data (n=2). Effects of the diets were evaluated using 3 way ANOVA analysis with Holm-Sidak method.

Three-way analysis of variance (ANOVA) on HMG-CoA reductase mRNA level

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	0.0375	0.0375	0.375	0.55
Type of fat (B)	1	0.0173	0.0173	0.173	0.683
-/+ Cholesterol (C)	1	0.202	0.202	2.019	0.176
A X B	1	0.156	0.156	1.564	0.23
A X C	1	0.0221	0.0221	0.222	0.645
B X C	1	0.0476	0.0476	0.476	0.501
A X B X C	1	0.0513	0.0513	0.513	0.485
Residual	15	1.499	0.0999		
Total	22	2.143	0.0974		



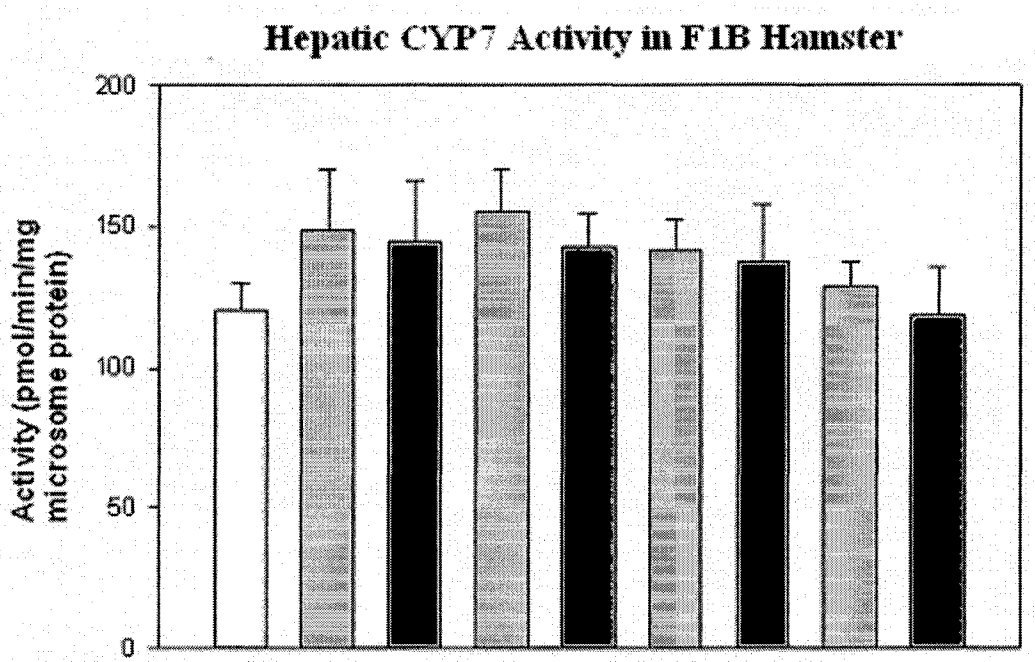
Fat Level	Low fat (5%)				High fat (20%)			
Dietary type	FO		MIX		FO		MIX	
Cholesterol	-	+	-	+	-	+	-	+

Figure 3.3 The Dietary Effects on The Expression of Hepatic CYP7 Activity in F1B Hamsters.

The hepatic CYP7 activity in F1B hamsters fed various diets was suppressed by the increased amount of fat in the diets. The types of the dietary fats and cholesterol supplement had no effect on the activity. The animals were fed either a fish oil supplemented diet or a control diet supplemented with a mixture of lard and MIX diet, at low fat (5%, w/w) or high fat (20%) levels, in the presence of 0.1% (Grey bars) or 0.25 % (Black bars) (w/w) cholesterol, respectively, for two weeks. Mean values are shown, with standard deviations indicated by vertical bars (n=6 at low fat diet, n=5 at high fat diets). Effects of the diets were evaluated using 3 way ANOVA analysis with Holm-Sidak method.

Three-way analysis of variance (ANOVA) on CYP7 activity

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	2722.766	2722.766	9.894	**0.003**
Type of fat (B)	1	442.726	442.726	1.609	0.213
-/+ Cholesterol (C)	1	616.885	616.885	2.242	0.143
A X B	1	862.389	862.389	3.134	0.085
A X C	1	5.81	5.81	0.0211	0.885
B X C	1	162.183	162.183	0.589	0.448
A X B X C	1	3.636	3.636	0.0132	0.909
Residual	35	9631.645	275.19		
Total	42	14458.421	344.248		



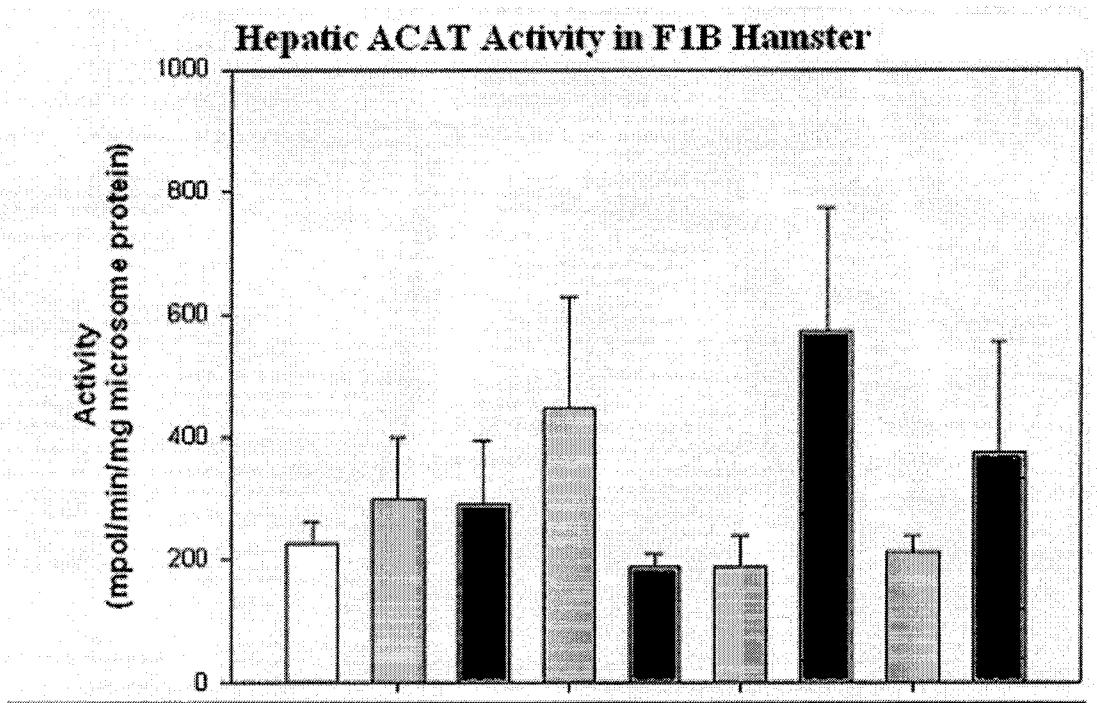
Fat level	—	Low fat (5%)				High fat (20%)			
Dietary type	control	FO		MIX		FO		MIX	
Cholesterol	—	—	+	—	+	—	+	—	+

Figure 3.4 The Dietary Effects on The Hepatic ACAT Activity in F1B Hamster

Overall, dietary cholesterol induced a significant increase on the hepatic ACAT activity ($P<0.05$). Moreover, this regulation of dietary cholesterol has a combination effect with the proportion and types of the dietary fats respectively ($P<0.001$ and $P<0.05$ respectively). The animals were fed either a fish oil supplemented diet or a control diet supplemented with a mixture of lard and safflower-seed oil (MIX) diet, at low fat (5%, w/w) or high fat (20%) levels, in the presence of 0.1% (Grey bars) or 0.25 % (Black bars) (w/w) cholesterol respectively for two weeks. Mean values are shown, with standard deviations indicated by vertical bars (n=6 at low fat diet, n=5 at high fat diets). Differences between groups were evaluated using 3 way ANOVA analysis with Holm-Sidak method.

Three-way analysis of variance (ANOVA) on ACAT activity

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	15397.76	15397.76	1.2	0.283
Type of fat (B)	1	17380.372	17380.372	1.354	0.255
-/+ Cholesterol (C)	1	56267.971	56267.971	4.385	**0.046**
A X B	1	14342.023	14342.023	1.118	0.3
A X C	1	307650.603	307650.603	23.975	**<0.001**
B X C	1	90094.272	90094.272	7.021	**0.014**
A X B X C	1	217.173	217.173	0.0169	0.897
Residual	26	333640.038	12832.309		
Total	33	839526.738	25440.204		



Fat level	—	Low fat (5%)				High fat (20%)			
Dietary type	control	FO		MIX		FO		MIX	
Cholesterol	—	—	+	—	+	—	+	—	+

diets appeared to be regulated differently when the animals were fed low fat diets, resulting in reduction of the activity, especially in animals fed MIX diets.

The analysis also shows a significant interaction between dietary cholesterol and the types of the dietary fat ($P<0.05$). In the presence of additional cholesterol, the ACAT activity was higher when the hamsters were fed fish oil diets (n-3 rich), compared to animals fed MIX diets (n-6 rich). Without additional dietary cholesterol, the MIX diets appeared to raise the activity slightly higher than animals fed fish oil diets. It is also interesting that the ACAT activity in microsomes from hamsters fed a chow diet approximates that for animals fed the low fat fish oil diet.

3.6 The Dietary Effects on The Expression of Hepatic SREBP-2 in F1B Hamsters

Here we used two antibodies N-19 and 1C6, which are against the N-and C-terminal respectively of the SREBP-2, to evaluate the expression of the mature and precursor forms of the protein. The band's intensity was normalized with the band of low fat fish oil diet in the same Western gel. Figure 3.5 shows the Western picture of the hamster liver lysate running in the same gel, but using antibodies N-19 (left) and 1C6 (right) respectively. Interestingly we found that the 1C6 might cross-react with the mature form of the SREBP-2, because the right picture also has a series of bands with molecular weight (~70 KDa) very close to the bands of mature form of SREBP-2 using antibody N-19. Therefore, we also quantitated those bands as mature SREBP-2. In Figure 3.6 and Figure 3.7, the variations of the diets had no significant influence on the expression of either the precursor or mature forms of the hepatic SREBP-2 in F1B hamsters. However, in Figure 3.7, the precursor forms are significantly reduced when the animals were fed

Figure 3.5 The Cross-Reaction of Antibody 1C6 on The Mature SREBP-2

This figure shows that the antibody 1C6 might cross-react with the mature form of the SREBP-2 by showing bands very close to the position (68 KDa) where the N-19 attached to the mature form, when the proteins were running in the same gel but developed separately.

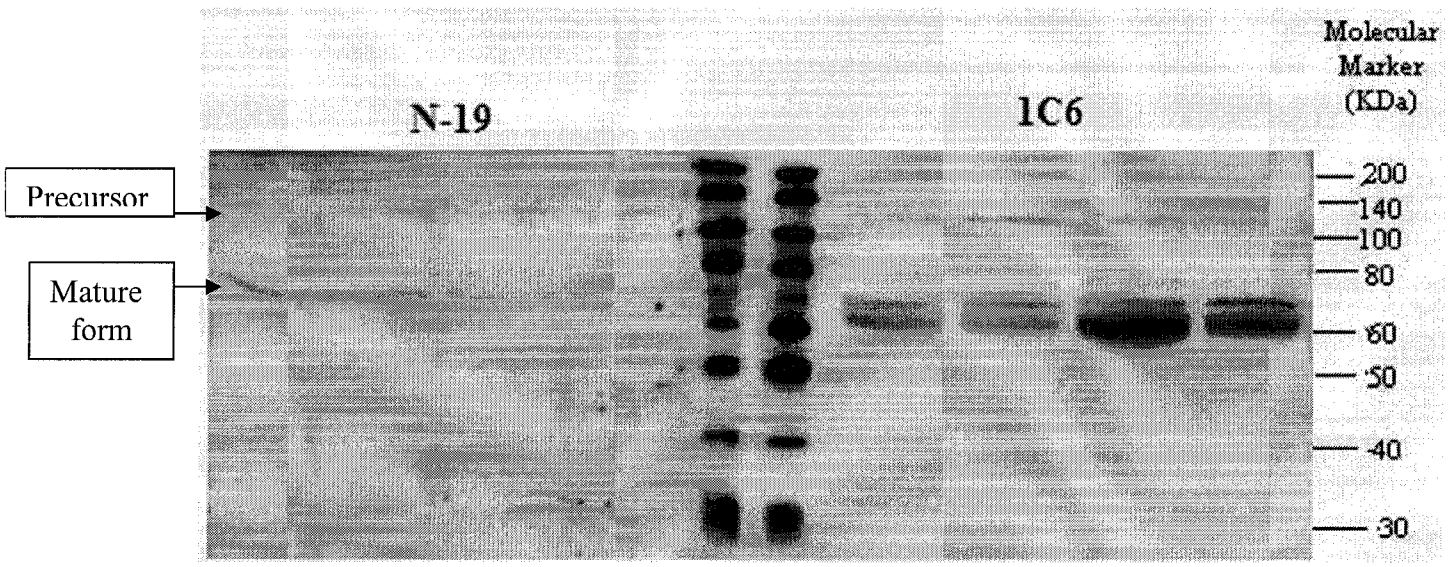
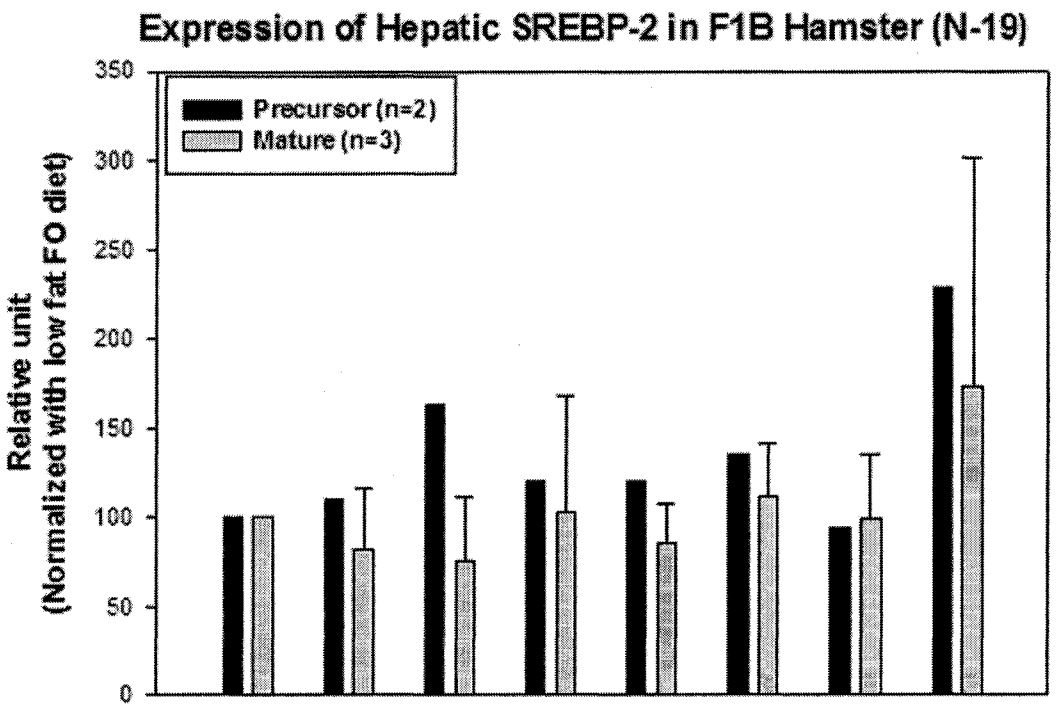

N-19
1C6
Molecular Marker (KDa)
Precursor
Mature form
200
140
100
80
60
50
40
30

Figure 3.6 The Dietary Effects on the Expression of SREBP-2 Using Antibody N-19

The diets had no significant effect on the expression of either the precursor or mature forms of SREBP-2 using antibody N-19. The animals were fed either a fish oil supplemented diet or a control diet supplemented with a mixture of lard and safflower-seed oil (MIX) diet, at low fat (5%, w/w) or high fat (20%) levels, in the presence of 0.1% or 0.25 % (w/w) cholesterol respectively for two weeks. The black and grey bars represent the precursor and mature forms of SREBP-2. The lack of the error bar is due to n=2 of the precursor data, but n=3 in mature form data. Mean values are shown, with standard deviations indicated by vertical bars (n=3). Differences between groups were evaluated using 3 way ANOVA analysis with Holm-Sidak method.

Three-way analysis of variance (ANOVA) on SREBP-2 using N-19

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	4443.696	4443.696	1.367	0.259
Type of fat (B)	1	1922.643	1922.643	0.592	0.453
-/+ Cholesterol (C)	1	4388.068	4388.068	1.35	0.262
A X B	1	2374.316	2374.316	0.731	0.405
A X C	1	3047.673	3047.673	0.938	0.347
B X C	1	3214.988	3214.988	0.989	0.335
A X B X C	1	4.659	4.659	0.00143	0.97
Residual	16	52000.158	3250.01		
Total	23	71396.201	3104.183		



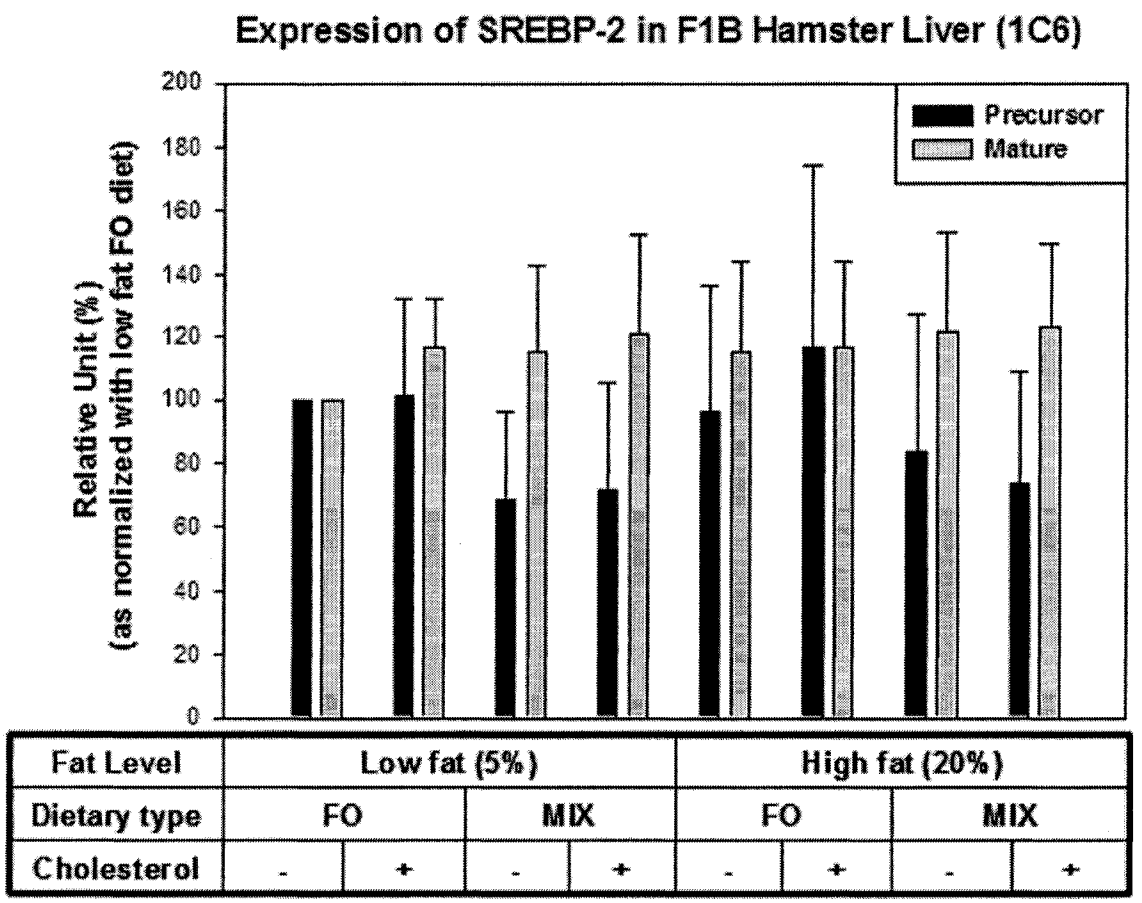
Fat Level	Low fat (5%)				High fat (20%)			
Dietary type	FO		MIX		FO		MIX	
Cholesterol	-	+	-	+	-	+	-	+

Figure 3.7 The Dietary Effects on the Expression of The SREBP-2 Using Antibody 1C6

The diets had no significant effect on the expression of mature forms of SREBP-2 using antibody 1C6, however the precursors were significantly reduced when the animals were fed MIX diets as compared to the FO diet groups. The animals were fed either a fish oil supplemented diet or a control diet supplemented with a mixture of lard and safflower-seed oil (MIX) diet, at low fat (5%, w/w) or high fat (20%) levels, in the presence of 0.1% or 0.25 % (w/w) cholesterol respectively for two weeks. Mean values are shown, with standard deviations indicated by vertical bars (n=3). Effects of the diets were evaluated using 3 way ANOVA analysis with Holm-Sidak method.

Three-way analysis of variance (ANOVA) on SREBP-2 using 1C6

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	218.844	218.844	0.332	0.572
Type of fat (B)	1	391.806	391.806	0.594	0.452
-/+ Cholesterol (C)	1	229.467	229.467	0.348	0.563
A X B	1	21.346	21.346	0.0324	0.859
A X C	1	137.277	137.277	0.208	0.654
B X C	1	39.344	39.344	0.0597	0.81
A X B X C	1	45.941	45.941	0.0697	0.795
Residual	16	10546.212	659.138		
Total	23	11630.238	505.663		


Expression of SREBP-2 in F1B Hamster Liver (1C6)
Relative Unit (%)
(as normalized with low fat FO diet)
200
180
160
140
120
100
80
60
40
20
0
Precursor
Mature
Fat Level
Low fat (5%)
High fat (20%)
Dietary type
FO
MIX
FO
MIX
Cholesterol
-
+
-
+
-
+
-
+

the MIX diets compared to those fed the FO diets. The reason why different antibodies showed different expression of the precursor is not clear.

Due to the insufficient data number, the data were pooled together in three ways, FO versus MIX diet, low fat versus high fat diets, without versus with dietary cholesterol, and analyzed by one way Anova. The analysis results show that the variations of the diets had no significant effect on the SREBP-2 expression.

3.7 The Dietary Effect on The Hepatic Microsome Cholesterol Concentration in F1B Hamster

Figure 3.8 shows the cholesterol concentration in the liver microsome of F1B hamsters fed various diets for two weeks. The additional cholesterol supplement alone had no impact on the microsomal cholesterol concentration. On the other hand, the types and the amount of the dietary fats individually had a significant effect ($P<0.05$ and $P<0.01$ respectively) on the cholesterol levels in this organelle. The results show that the high fat or the fish oil (n-3 rich) diets increased the cholesterol concentration in the hepatic microsomes of the hamsters.

The statistical analysis also shows a significant interaction between the dietary cholesterol and the amount of the dietary fats ($P<0.05$), resulting in an elevated concentration of cholesterol when hamsters were fed cholesterol and high fat diets, compared to animals fed high fat diets without dietary cholesterol. However, when hamsters were fed with low fat diets, the effect of dietary cholesterol on the levels of microsomal cholesterol was not as great as that seen in high fat groups, and was even lower in animals fed low fat MIX diet.

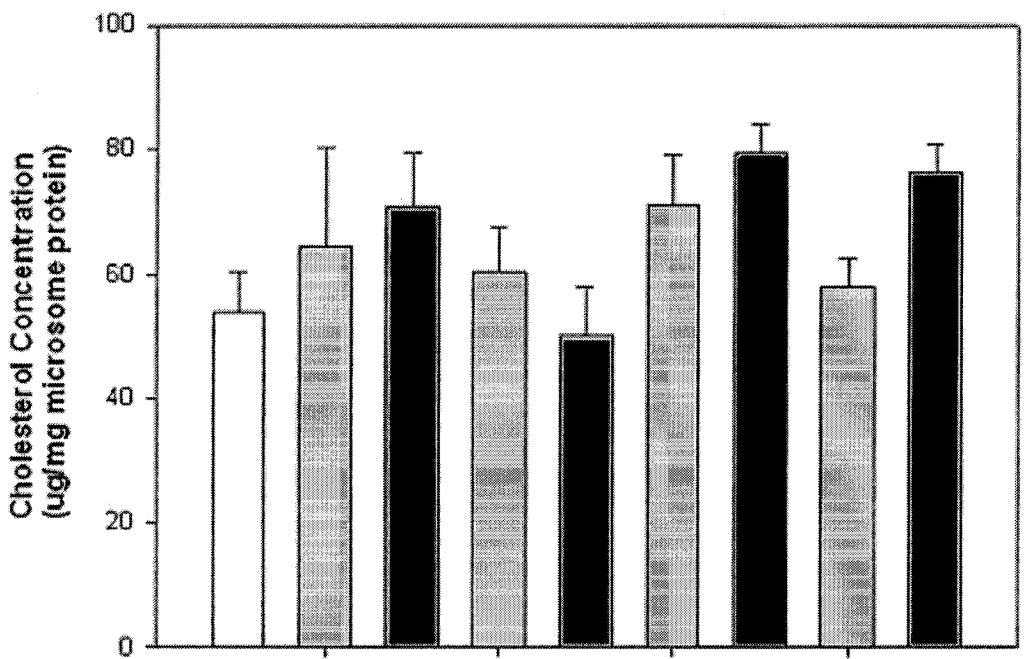
Fig 3.8 The Dietary Effects on The Microsome Cholesterol Concentration in F1B Hamsters

The high fat and the n-3 rich diets individually induced a significant increase of the concentration of microsomal cholesterol in F1B hamsters. The dietary cholesterol had no significant effect on the microsomal cholesterol. The statistical analysis also showed a significant interaction between dietary cholesterol and high fat diets, resulting in increased microsomal cholesterol in the hamsters. The animals were fed either a fish oil supplemented diet or a control diet supplemented with a mixture of lard and safflower-seed oil (MIX) diet, at low fat (5%, w/w) or high fat (20%) levels, in the presence of 0.1% (Grey bars) or 0.25 % (Black bars) (w/w) cholesterol respectively for two weeks. Mean values are shown, with standard deviations indicated by vertical bars (n=6 at low fat diet, n=5 at high fat diets). Effects of the diets were evaluated using 3 way ANOVA analysis with Holm-Sidak method.

Three-way analysis of variance (ANOVA) on microsomal cholesterol level

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	558.653	558.653	7.945	**0.012**
Type of fat (B)	1	631.057	631.057	8.974	**0.009**
-/+ Cholesterol (C)	1	176.911	176.911	2.516	0.132
A X B	1	25.922	25.922	0.369	0.552
A X C	1	347.383	347.383	4.94	**0.041**
B X C	1	16.781	16.781	0.239	0.632
A X B X C	1	259.524	259.524	3.691	0.073
Residual	16	1125.091	70.318		
Total	23	3141.321	136.579		

Hepatic Microsome Cholesterol Concentration in F1B Hamster



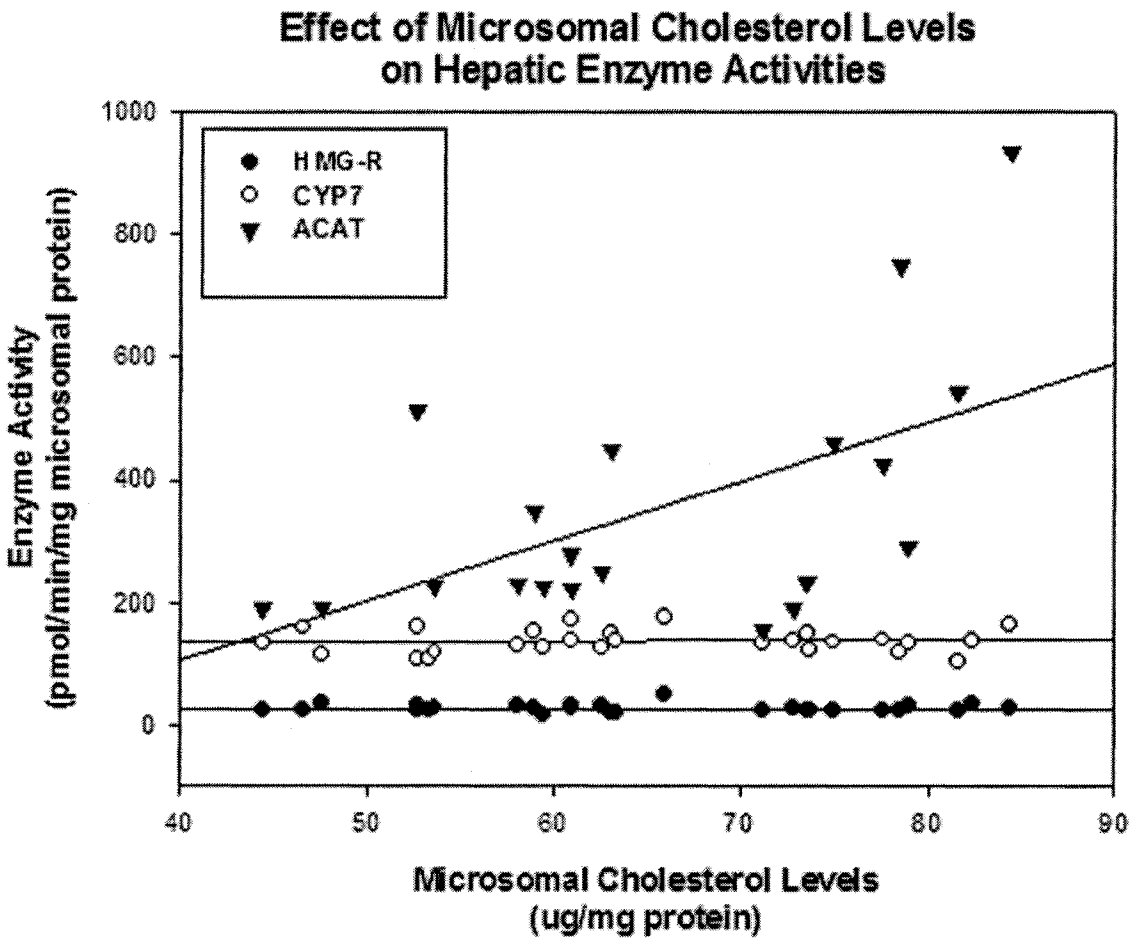
Fat level	—	Low fat (5%)				High fat (20%)			
Dietary type	control	FO		MIX		FO		MIX	
Cholesterol	—	—	+	—	+	—	+	—	+

Figure 3.9 shows a regression analysis between the microsomal cholesterol and the activities of the three microsomal enzymes, HMG-CoA reductase, CYP7, and ACAT, in F1B hamsters fed different diets for two weeks. The data for the activities and the concentration of microsomal cholesterol were obtained within the same animals. The activities of HMG-CoA reductase and the CYP7 had no correlation with the microsomal cholesterol ($R^2 \approx 0$). The ACAT activity, on the other hand, showed a significant correlation with the concentration of the microsomal cholesterol ($P<0.05$; $R^2 = 0.31$).

When comparing Figure 3.4 and Figure 3.8, we found that the variations of the diets had a similar impact on both the ACAT activity and the concentration of the microsomal cholesterol in F1B hamster liver, which showed a greater influence on ACAT activity than on cholesterol concentration. This may suggest that the increased cholesterol concentration, caused by the dietary variation, in the hepatic microsome of the hamsters greatly promotes the ACAT activity, which results in only a slight increase of cholesterol in the hepatic microsome of F1B hamsters.

Figure 3.9 The Effect of Microsomal Cholesterol Concentration on The Hepatic Enzyme Activities in F1B Hamster.

The concentration of the microsomal cholesterol was significantly correlated ($P<0.05$, $R^2 \approx 0.31$) with the microsomal ACAT activity, but not with HMG-CoA reductase and CYP7, in F1B hamster liver. A regression analysis was performed to evaluate the correlation between the microsomal cholesterol concentration and the activities of the enzymes, HMG-CoA reductase, CYP7, and ACAT. The data of the activity and the concentration of microsomal cholesterol were obtained within the identical animals. The black dot, white dot, and the triangle represent the specific activity of HMG-CoA reductase, CYP7, and ACAT, respectively, of the same animal with corresponding cholesterol concentration in the hepatic microsome.


Effect of Microsomal Cholesterol Levels
on Hepatic Enzyme Activities
Enzyme Activity
(pmol/min/mg microsomal protein)
1000
800
600
400
200
0
HMG-R
CYP7
ACAT
40
50
60
70
80
90
Microsomal Cholesterol Levels
(ug/mg protein)

3.8 The Dietary Effects on The Microsomal Fatty Acids Composition in F1B Hamster Liver

Figure 3.10 shows the fatty acid composition of total microsomal lipids in the livers of F1B hamsters fed different diets for two weeks. The total fatty acids were separated into 3 groups, saturated fatty acids (SATs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) for simple illustration. The variations of the diets had no significant influence on the proportion of the MUFAs and PUFAs out of total fatty acids in the hepatic microsome of the hamsters. The high fat diets, however, induced a significant increase ($P<0.05$) in the proportion of the total SATs in the microsome. Interestingly, we found that the proportion of the total MUFAs and PUFAs was much lower and higher, respectively, in the hamsters fed the control diet, compared with those fed experimental diets, which is not what we had expected.

Figure 3.11 shows the percentage of only microsomal n-3 and n-6 PUFAs in F1B hamster liver. When comparing among the three dietary factors individually, only the n-3/n-6 ratio in the diet had a significant impact ($P<0.05$) on the amount of n-3 and n-6 in the hepatic microsome of the hamsters. There was an increase of n-3 PUFAs in the microsome when the animals were fed fish oil diets (n-3 rich), and an increased n-6 PUFAs when the animals were fed the MIX (n-6 rich) diets.

Figure 3.12 shows the ratio of the n-3 and n-6 PUFAs in the hepatic microsome of F1B hamsters fed various diets for two weeks. Only the dietary n-3/n-6 ratio in the diets had a significant influence ($P<0.001$) on the n-3/n-6 PUFAs ratio in the microsome. The ratio is high when the hamsters were fed fish oil diet, and low when feeding hamsters with MIX diets, regardless of the amount of the dietary fat and cholesterol in the diets.

Figure 3.13 shows a significant interaction between the cholesterol concentration and the content of n-3 PUFAs in the hepatic microsome in F1B hamsters fed various diets for two weeks ($P<0.05$, $R^2= 0.224$). This figure indicates that the content of n-3 PUFAs in the microsome was not only affected by the types of dietary PUFAs (shown in Figure 3.12), but also correlated with the cholesterol levels in the microsome. It is suggested that when the cholesterol levels in the microsome increases due to the diet, more n-3 PUFAs were needed for incorporation with cholesterol in the hepatic microsome of F1B hamsters. This positive correlation may contribute to stabilize the environment of the microsome for proper enzyme activation.

3.9 The Correlation of Microsomal n-3 Fatty Acids and Enzyme Activities

Both Figure 3.14 and 3.15 show a positive correlstion between the content of microsomal n-3 and hepatic HMG-CoA reductase ($P<0.05$, $R^2=0.282$), and ACAT activity ($P<0.05$; $R^2= 0.334$) in the F1B hamster fed various diets for two weeks. Together, these results show that although the activity of HMG-CoA reductase and ACAT were not regulated by the dietary fat directly, both activities are positively correlated with the level of n-3 fatty acids in the hepatic microsome, which suggests that the microsomal environment may play a much more important role on cholesterol homeostasis rather than the diet in F1B hamster.

Figure 3.10 The Dietary Effects on The Microsomal Fatty Acids Composition in F1B Hamster Liver

The diets had no significant influence on the proportion of the MUFAs and PUFAs as a proportion of total fatty acids in the hepatic microsomes of the F1B hamsters. The high fat diets, however, induced a significant increase ($P<0.05$) in the proportion of the total SATs in the microsomes. Interestingly, we found that the proportions of the total MUFAs and PUFAs were much smaller and higher respectively in the hamsters fed the control diet, compared to those fed experimental diets.

The total fatty acids were separated into 3 groups, saturated fatty acids (SATs; black bars), monounsaturated fatty acids (MUFAs; dark grey bars), and polyunsaturated fatty acids (PUFAs; light grey bars) for simple illustration.

The animals were fed either a fish oil supplemented diet or a control diet supplemented with a mixture of lard and safflower-seed oil (MIX) diet, at low fat (5%, w/w) or high fat (20%) levels, in the presence of 0.1% or 0.25 % (w/w) cholesterol respectively for two weeks. Mean values are shown, with standard deviations indicated by vertical bars (n=3). Effects of the diets were evaluated using 3 way ANOVA analysis with Holm-Sidak method.

Three-way analysis of variance (ANOVA) on microsomal SAT level

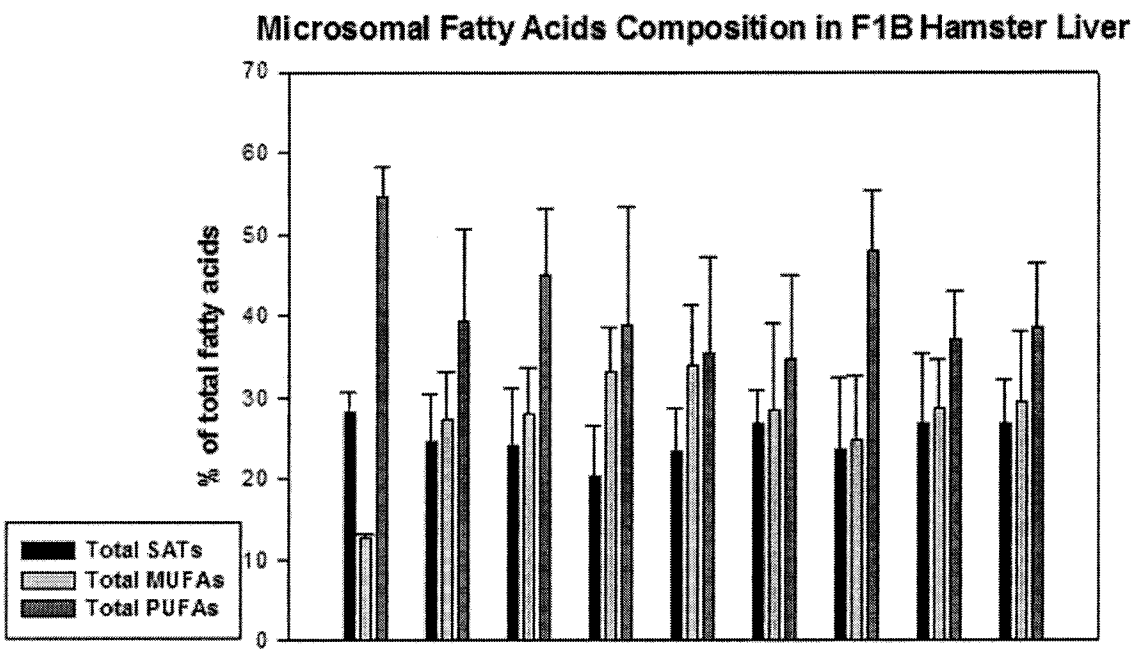
Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	59.945	59.945	1.376	0.255
Type of fat (B)	1	1.493	1.493	0.0343	0.855
-/+ Cholesterol (C)	1	0.18	0.18	0.00413	0.949
A X B	1	29.327	29.327	0.673	0.422
A X C	1	13.214	13.214	0.303	0.588
B X C	1	19.289	19.289	0.443	0.513
A X B X C	1	0.0721	0.0721	0.00166	0.968
Residual	20	871.11	43.555		
Total	27	990.499	36.685		

Three-way analysis of variance (ANOVA) on microsomal MUFA level

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	52.5	52.5	0.941	0.344
Type of fat (B)	1	126.195	126.195	2.263	0.148
-/+ Cholesterol (C)	1	0.905	0.905	0.0162	0.9
A X B	1	22.159	22.159	0.397	0.536
A X C	1	7.845	7.845	0.141	0.712
B X C	1	8.725	8.725	0.156	0.697
A X B X C	1	7.895	7.895	0.142	0.711
Residual	20	1115.399	55.77		
Total	27	1341.257	49.676		

Three-way analysis of variance (ANOVA) on microsomal PUFA level

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	0.141	0.141	0.0014	0.971
Type of fat (B)	1	120.393	120.393	1.191	0.288
-/+ Cholesterol (C)	1	119.977	119.977	1.187	0.289
A X B	1	4.355	4.355	0.0431	0.838
A X C	1	67.881	67.881	0.672	0.422
B X C	1	186.436	186.436	1.845	0.189
A X B X C	1	4.26	4.26	0.0422	0.839
Residual	20	2020.905	101.045		
Total	27	2504.781	92.77		



Fat level	—	Low fat (5%)				High fat (20%)			
Dietary type	control	FO		MIX		FO		MIX	
Cholesterol	—	—	+	—	+	—	+	—	+

Figure 3.11 The Dietary Effects on The n-3 And n-6 PUFAs in the Hepatic Microsome of F1B Hamster

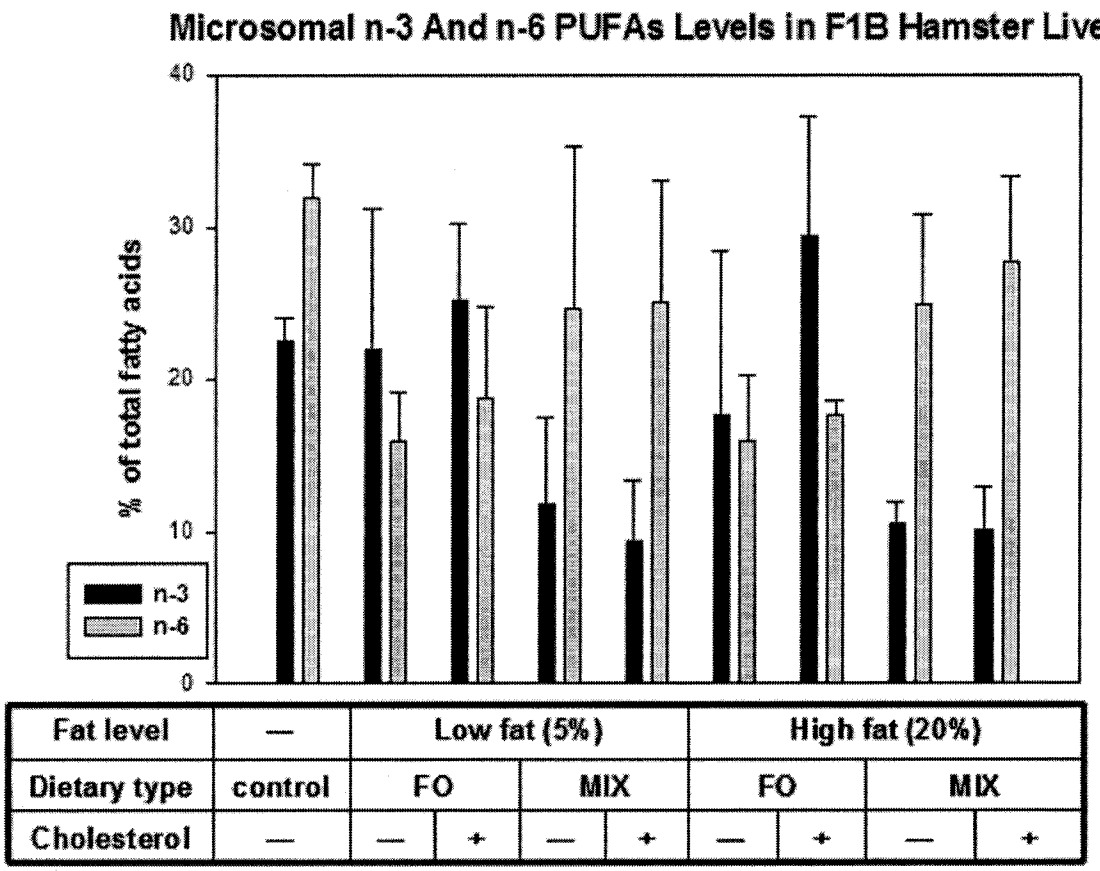
When comparing among the three dietary factors individually, only the type of the dietary fats (n-3 or n-6 rich) had a significant impact on the amount of n-3 and n-6 ($P<0.01$) in the hepatic microsomes of the hamsters. It was accompanied by an increase in n-3 PUFAs in the microsomes when the animals were fed fish oil diets (n-3 rich), and an n-6 PUFAs increase when the animals were fed the MIX (n-6 rich) diets. The animals were fed either a fish oil supplemented diet or a control diet supplemented with a mixture of lard and safflower-seed oil (MIX) diet, at low fat (5%, w/w) or high fat (20%) levels, in the presence of 0.1% (Grey bars) or 0.25 % (Black bars) (w/w) cholesterol respectively for two weeks. Mean values are shown, with standard deviations indicated by vertical bars (n=3). Effects of the diets were evaluated using 3 way ANOVA analysis with Holm-Sidak method.

Three-way analysis of variance (ANOVA) on microsomal n-3 fatty acids level

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	0.209	0.209	0.00449	0.947
Type of fat (B)	1	1168.687	1168.687	25.096	**<0.001**
-/+ Cholesterol (C)	1	66.518	66.518	1.428	0.246
A X B	1	0.0779	0.0779	0.00167	0.968
A X C	1	46.832	46.832	1.006	0.328
B X C	1	138.162	138.162	2.967	0.1
A X B X C	1	19.57	19.57	0.42	0.524
Residual	20	931.361	46.568		
Total	27	2311.529	85.612		

Three-way analysis of variance (ANOVA) on microsomal n-6 fatty acids level

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	1.181	1.181	0.0313	0.861
Type of fat (B)	1	500.215	500.215	13.253	**0.002**
-/+ Cholesterol (C)	1	25.123	25.123	0.666	0.424
A X B	1	6.999	6.999	0.185	0.671
A X C	1	0.705	0.705	0.0187	0.893
B X C	1	0.59	0.59	0.0156	0.902
A X B X C	1	5.396	5.396	0.143	0.709
Residual	20	754.844	37.742		
Total	27	1311.781	48.584		



Fat level	—	Low fat (5%)				High fat (20%)			
Dietary type	control	FO		MIX		FO		MIX	
Cholesterol	—	—	+	—	+	—	+	—	+

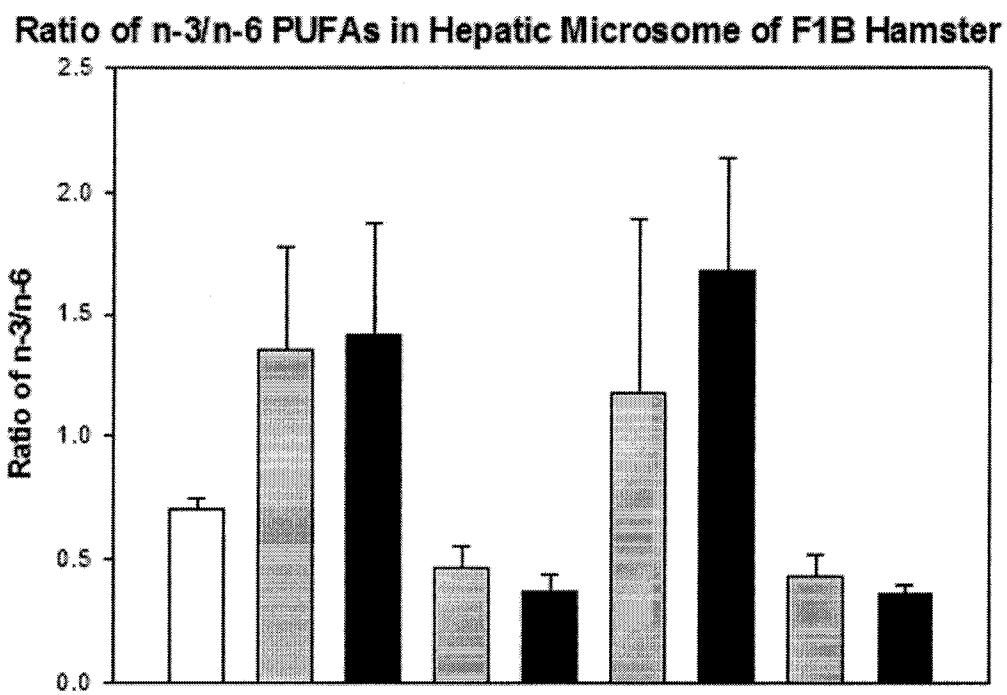
Figure 3.12 The Dietary Effects on The Ratio of n-3/n-6 PUFAs in the Hepatic Microsome of F1B Hamster

The ratio of n-3/n-6 PUFAs in the hepatic microsomes was significantly increased ($P<0.001$) when feeding fish oil diets to the F1B hamsters, regardless of the amount of dietary fat or cholesterol. The ratio was reduced when the hamsters were fed the MIX diets.

The animals were fed either a fish oil supplemented diet or a control diet supplemented with a mixture of lard and safflower-seed oil (MIX) diet, at low fat (5%, w/w) or high fat (20%) levels, in the presence of 0.1% (Grey bars) or 0.25 % (Black bars) (w/w) cholesterol respectively for two weeks. Mean values are shown, with standard deviations indicated by vertical bars (n=3). Effects of the diets were evaluated using 3 way ANOVA analysis with Holm-Sidak method.

Three-way analysis of variance (ANOVA) on the ratio of microsomal n-3/n-6 fatty acids

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	0.000881	0.000881	0.00599	0.939
Type of fat (B)	1	6.876	6.876	46.738	**<0.001**
-/+ Cholesterol (C)	1	0.0651	0.0651	0.443	0.514
A X B	1	0.00691	0.00691	0.047	0.831
A X C	1	0.09	0.09	0.612	0.443
B X C	1	0.235	0.235	1.599	0.221
A X B X C	1	0.0731	0.0731	0.497	0.489
Residual	20	2.942	0.147		
Total	27	10.157	0.376		



Fat level	—	Low fat (5%)				High fat (20%)			
Dietary type	control	FO		MIX		FO		MIX	
Cholesterol	—	—	+	—	+	—	+	—	+

Figure 3.13 The Correlation Between the Cholesterol Concentration And the n-3 PUFAs in the Hepatic Microsome of F1B Hamster

Regression analysis between the cholesterol concentration and the n-3 PUFAs in the hepatic microsomes of F1B hamsters indicated a significant positive correlation between the cholesterol concentration and the n-3 PUFAs in the hepatic microsomes in the hamsters fed various diets for two weeks ($P<0.05$; $R^2= 0.224$).

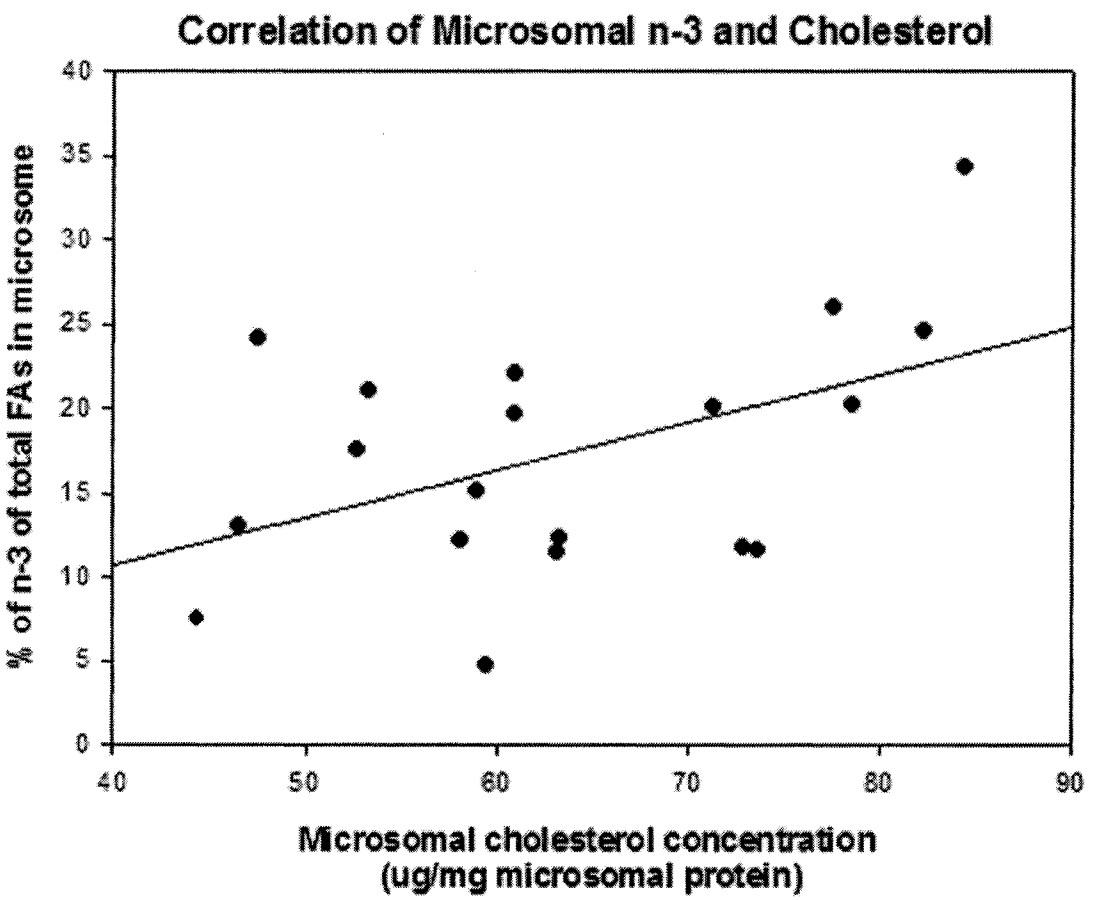

Correlation of Microsomal n-3 and Cholesterol
% of n-3 of total FAs in microsome
0
5
10
15
20
25
30
35
40
40
50
60
70
80
90
Microsomal cholesterol concentration
(ug/mg microsomal protein)

Figure 3.14 The Correlation Between the HMG-CoA Reductase Activity And the n-3 PUFAs in the Hepatic Microsome of F1B Hamster

A regression analysis between the HMG-CoA reductase activity and the n-3 PUFAs in the hepatic microsomes of F1B hamsters indicated a significant positive correlation between the cholesterol concentration and the n-3 PUFAs in the hepatic microsomes in hamsters fed various diets for two weeks ($P<0.05$; $R^2= 0.282$).

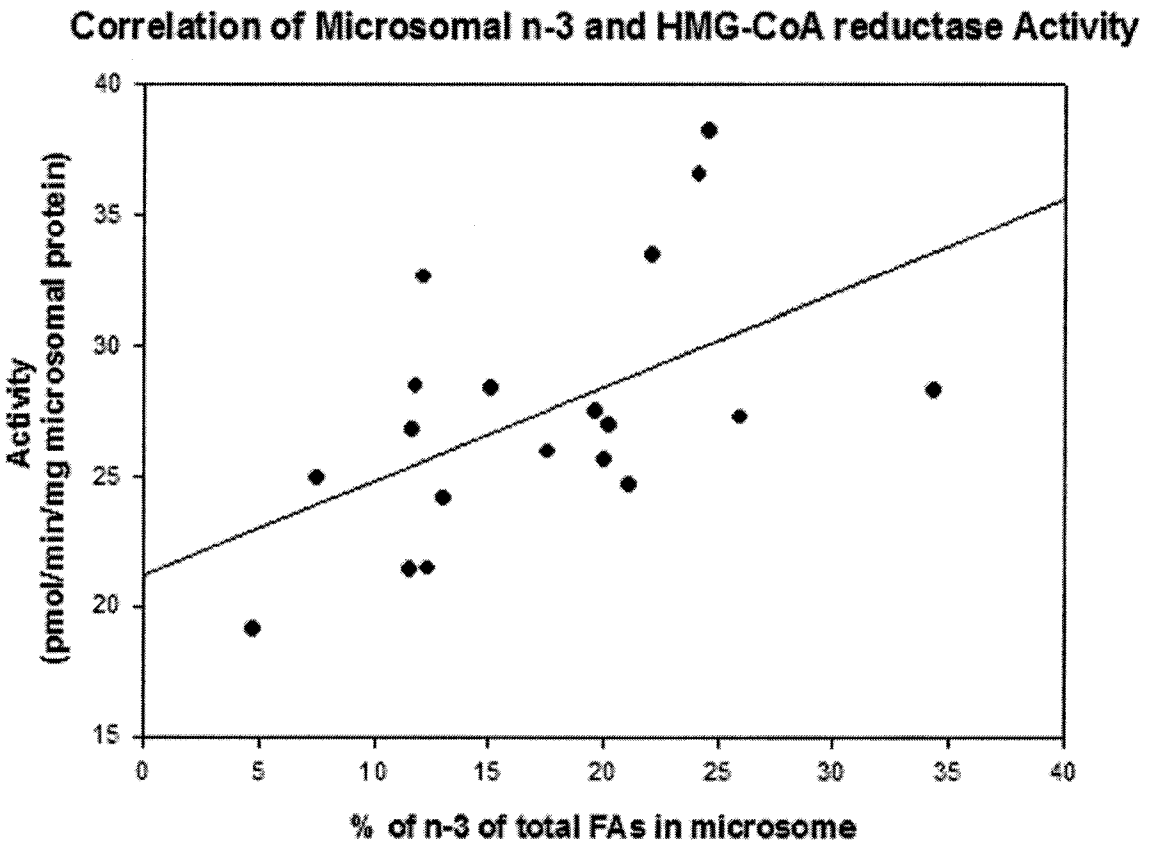
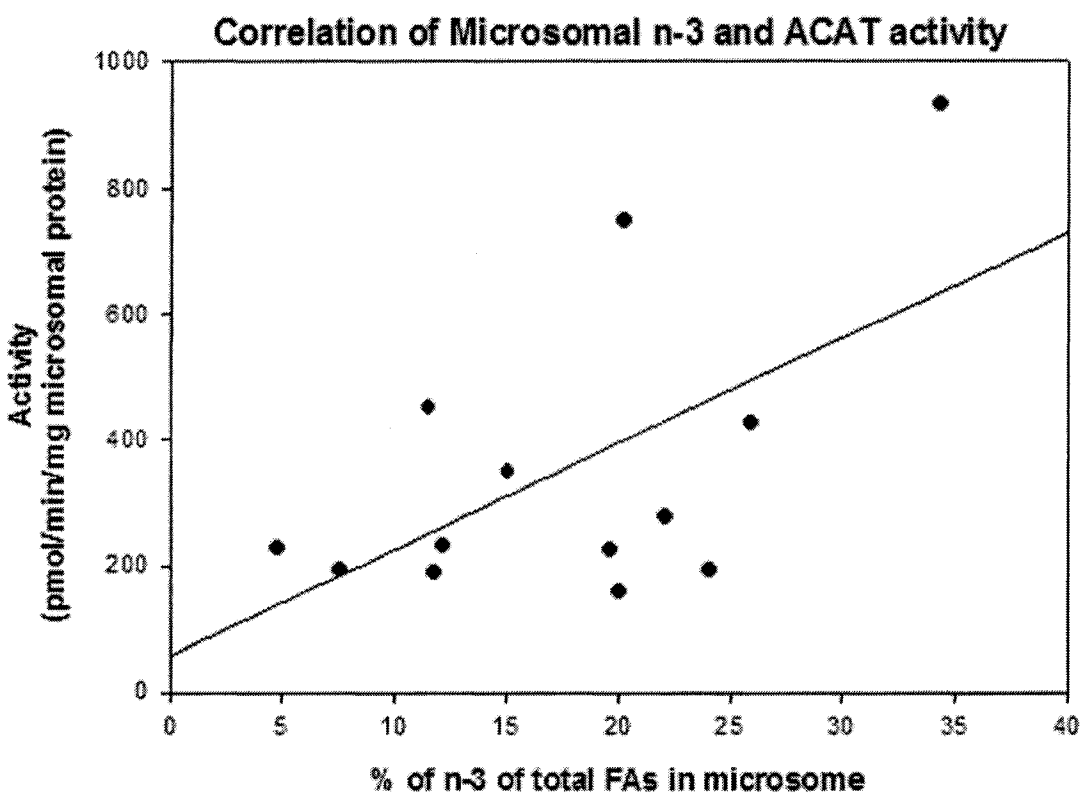

Correlation of Microsomal n-3 and HMG-CoA reductase Activity
Activity
(pmol/min/mg microsomal protein)
40
35
30
25
20
15
0
5
10
15
20
25
30
35
40
% of n-3 of total FAs in microsome

Figure 3.15 The Correlation Between the ACAT Activity And the n-3 PUFAs in the Hepatic Microsome of F1B Hamster

Regression analysis between the ACAT activity and the n-3 PUFAs in the hepatic microsomes of F1B hamsters indicated a significant positive correlation between the cholesterol concentration and the n-3 PUFAs in the hepatic microsomes in the hamsters fed various diets for two weeks ($P<0.05$; $R^2= 0.334$).


Correlation of Microsomal n-3 and ACAT activity
Activity
(pmol/min/mg microsomal protein)
1000
800
600
400
200
0
0
5
10
15
20
25
30
35
40
% of n-3 of total FAs in microsome

3.10 The Dietary Effects on The Hepatic Microsome Phospholipid Composition in F1B Hamster

Figure 3.16 shows the percentage of phospholipid composition out of total phospholipids of the hepatic microsome in F1B hamsters. The differences due to diets were not significant.

Figure 3.16 The Dietary Effects on The Microsomal Phospholipid Composition in F1B Hamster Liver

The effect of diet on the phospholipid composition in the microsome was not significant. The phospholipids are separated into three groups, phosphatidylethanolamine (PE; black bars), phosphatidylserine (PS; dark grey bars), and phosphatidylcholine plus sphingomyelin (PC+SPH; light grey bars). The figure shows the percentage of phospholipid composition out of total phospholipids of the hepatic microsome in F1B hamsters.

The animals were fed either a fish oil supplemented diet or a control diet supplemented with a mixture of lard and safflower-seed oil (MIX) diet, at low fat (5%, w/w) or high fat (20%) levels, in the presence of 0.1% or 0.25 % (w/w) cholesterol respectively for two weeks. Mean values are shown, with standard deviations indicated by vertical bars (n=3), and the lack of the error bar is due to insufficient data number (n=2). Differences between groups were evaluated using 3 way ANOVA analysis with Holm-Sidak method.

Three-way analysis of variance (ANOVA) on microsomal PE

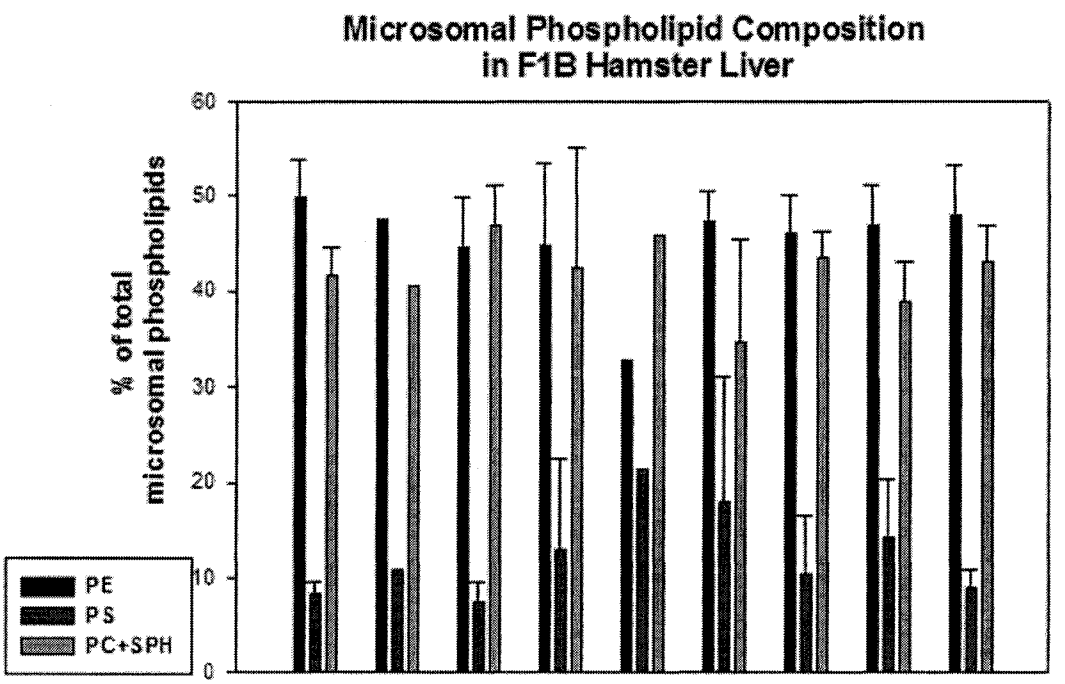
Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	117.813	117.813	4.363	0.055
Type of fat (B)	1	56.333	56.333	2.086	0.171
-/+ Cholesterol (C)	1	76.339	76.339	2.827	0.115
A X B	1	86.761	86.761	3.213	0.095
A X C	1	70.406	70.406	2.607	0.129
B X C	1	14.52	14.52	0.538	0.475
A X B X C	1	43.828	43.828	1.623	0.223
Residual	14	378.067	27.005		
Total	21	747.939	35.616		

Three-way analysis of variance (ANOVA) on microsomal PS

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	0.188	0.188	0.00248	0.961
Type of fat (B)	1	35.938	35.938	0.475	0.502
-/+ Cholesterol (C)	1	20.367	20.367	0.269	0.612
A X B	1	155.28	155.28	2.053	0.174
A X C	1	108.601	108.601	1.436	0.251
B X C	1	65.489	65.489	0.866	0.368
A X B X C	1	29.978	29.978	0.396	0.539
Residual	14	1059.012	75.644		
Total	21	1451.133	69.102		

Three-way analysis of variance (ANOVA) on microsomal PC+SPH

Source of Variation	DF	SS	MS	F	P
Amount of fat (A)	1	84.978	84.978	0.897	0.36
Type of fat (B)	1	7.468	7.468	0.0789	0.783
-/+ Cholesterol (C)	1	178.384	178.384	1.884	0.192
A X B	1	3.557	3.557	0.0376	0.849
A X C	1	3.853	3.853	0.0407	0.843
B X C	1	18.089	18.089	0.191	0.669
A X B X C	1	0.963	0.963	0.0102	0.921
Residual	14	1325.897	94.707		
Total	21	1642.69	78.223		



Fat level	—	Low fat (5%)				High fat (20%)			
Dietary type	control	FO		MIX		FO		MIX	
Cholesterol	—	—	+	—	+	—	+	—	+

Chapter 4 Discussion

4.1 Dietary Fat and Cholesterol Homeostatsis

Since the studies of Bang and Dyerberg on the lipoprotein profile of Greenland Eskimos in the 1970's, fish oil and other oils rich in n-3 PUFA have been proposed to offer protection against hyperlipidemia and CHD (Bang and Dyerberg, 1972; Dyerberg *et al.*, 1975; Dyerberg *et al.*, 1978). However, despite decades of animal studies and large scale human trails, the only consistent effect of dietary n-3 PUFAs has been reduced triglyceride levels in hypertriglyceridemic subjects (Connor, 1988; Connor *et al.*, 1993; Hamazaki *et al.*, 2003). Moreover, there is growing concern that diets rich in n-3 PUFAs may have a negative impact on patients with hypercholesterolemia (Failor *et al.*, 1988; Hsu *et al.*, 2000). Lin et al. reported recently that in hamsters with a low plasma cholesterol level, addition of n-3 PUFA to the diet results in a significant reduction in total plasma cholesterol, and in both VLDL and LDL cholesterol compared with animals fed an n-6 PUFA diet (Lin *et al.*, 2005). However, when these animals were fed a diet containing 0.5% cholesterol (w/w), an n-3 diet group was more hypercholesterolemic than an n-6 diet group. This suggested that there was an interaction between the cholesterol mediated regulation of cholesterol metabolism and the regulation mediated by dietary fatty acids.

Our laboratory has done similar studies using the inbred F1B hamster strain. This animal model has been used extensively because of its susceptibility to diet induced hyperlipidemia and atherosclerotic lesions (Kowala *et al.*, 1991). An early study in our laboratory showed a clear difference between the response of the normal hamster to increased n-3/n-6 ratio in the diet and the response of the F1B strain to a similar change in the types of fatty acids in the diet. While Lin et al. (Lin *et al.*, 2005) reported that

replacement of n-6 PUFA in the diet of normal hamsters with n-3 PUFA reduced VLDL-cholesterol by more than 60%, a similar change in dietary fatty acids in the F1B hamster resulted in a 4-5 fold increase in VLDL-cholesterol.

When 0.5% (w/w) cholesterol was added to the diets of the normal hamster, there was a marked increase in the VLDL-cholesterol levels in the plasma, but in this case the n-3 diet resulted in a more hypercholesterolemic animal than the n-6 diet (Lin *et al.*, 2005). In contrast, addition of cholesterol to the diets of the F1B hamsters resulted in a very modest change in VLDL-cholesterol level in the plasma although the n-3 fed animals were still much more hypercholesterolemic than those fed n-6 PUFAs (de Silva *et al.*, 2004). These observations suggested that the regulation of cholesterol metabolism in the F1B hamster is different.

The present study is an extension of our previous study that sought to investigate the cholesterol metabolism in the F1B hamster caused by different dietary PUFAs and cholesterol at the hepatic cellular level. Our objective was to examine the regulation of cholesterol metabolism in the liver of F1B hamsters by both dietary cholesterol and the types and level of fatty acids in the diet. We looked at the expression of several microsome associated enzymes that play key roles in cholesterol metabolism in liver including, HMG-CoA reductase, CYP7, ACAT, a corresponding transcription factor, SREBP-2, and the lipid composition in the microsomes.

4.2 Dietary Effects on Microsomal Enzyme Activity

We found that dietary cholesterol down- and up-regulated the activity of HMG-CoA reductase and ACAT respectively, but had no effect on CYP7 activity. This inducing

effect of dietary cholesterol on hepatic ACAT activity was correlated with the amount of dietary fat and fat types. **(This suggests?)** We also found that the cholesterol levels in the microsomes were increased when the hamsters were fed high fat and cholesterol supplemented diets. Moreover, the n-3 PUFA level was positively correlated with elevated cholesterol level in the microsomes. Thus we suggested that the dietary effects on enzyme activity and the cellular cholesterol homeostasis may be regulated by the fluidity of the microsomal membrane and the theoretical cholesterol pool in F1B hamster.

4.2.1 Dietary Cholesterol and PUFAs on CYP7 Activity

The activity of CYP7, the initial enzyme in the classic pathways of bile acid synthesis, is tightly regulated at the transcriptional level, since the activity is highly correlated with its mRNA levels (Agellon and Torchia, 2000;Russell and Setchell, 1992). Many studies have shown that the CYP7 gene expression is suppressed by bile acids and induced by cholesterol (Chiang *et al.*, 1990;Chiang and Stroup, 1994).

However recent studies on African green monkeys and hyperlipidemic rabbits have shown that additional dietary cholesterol does not always stimulate the CYP7 activity as expected (Rudel *et al.*, 1994;Xu *et al.*, 1995). Studies on normal hamsters showed that the dietary unsaturated fats (linoleic acid and oleic acid) and saturated fat (palmitic acid) exert an opposite effect on the hepatic CYP7 activity when the hamsters were fed with 0.1% dietary cholesterol (Kurushima *et al.*, 1995;Kurushima *et al.*, 1995). Studies in mice showed that, although different types of dietary fatty acids (PUFA, MUFA, SFA) all increase the CYP7 activity, the PUFA induced the highest activity among the dietary fats. Interestingly, when an additional 1% cholesterol was supplemented in the diets, the

dietary cholesterol further induced the CYP7 activity in animals fed the PUFA diets, while the activities were suppressed by additional cholesterol in hamsters fed the MUFA, SFA diets (Cheema *et al.*, 1997). The results also showed that although the PUFA diets without dietary cholesterol induced the highest abundance on CYP7 mRNA, it was not associated with the highest activity. This fact implied that the dietary fats may regulate differently than at the transcriptional level.

In our study, the CYP7 activity, surprisingly, was not affected by dietary cholesterol when supplemented in the polyunsaturated fat diets. The CYP7 activity was only affected by the amount of dietary fat, resulting in a reduction of the activity when the hamsters were fed the high fat diets. The reason why dietary cholesterol showed no effect on the activity was not clear.

4.2.2 Dietary Cholesterol and PUFA on HMG-CoA Reductase Activtiy

The rate limited enzyme of cholesterol biosynthsis, HMG-CoA reductase, and its feedback regulation has been studied for many years in different animal models. The reductase activity was suggested to be regulated differently depending on the animal's susceptibility to dietary cholesterol. Dietary cholesterol exerted feedback regulation mainly at the posttranslational level in rats that are relatively resistant to dietary cholesterol (Ness *et al.*, 1991;Spady and Cuthbert, 1992) and at the transcriptional level in rabbits and hamsters that are relatively susceptible to dietary cholesterol (Gil *et al.*, 1986;Rudling, 1992). In our study, the hepatic HMG-CoA reductase activity was only affected by dietary cholesterol, resulting in a slight reduction of the activity when cholesterol was added to the diets. Although the extent of the reduction was not as great

as we expected and the mRNA levels were not affected by any of the dietary variations, these results suggested that the dietary cholesterol exerted the regulatory effect on the reductase activity at a level other than the transcription level. However, mRNA data include n=3, 2, so variability could be an issue.

Although studies in mice (Du *et al.*, 2003) and rats (Jossic-Corcos *et al.*, 2005) showed that n-3 PUFA rich diets suppressed the hepatic HMG-CoA reductase activity and mRNA respectively much more than the n-6 PUFA rich diets without dietary cholesterol supplement, studies on hamsters showed that addition of dietary unsaturated fatty acids (linoleic acid or oleic acid) or saturated fatty acids (palmitic acid) to 0.1% cholesterol-supplemented diets didn't alter the HMG-CoA reductase activityc in hamsters, compared with those fed cholesterol-supplemented diet (Kurushima *et al.*, 1995;Kurushima *et al.*, 1995). The results may suggest that the suppressing effect of dietary fat and cholesterol on the reductase activity is mainly due to the presence of cholesterol instead of dietary fats in hamsters.

Our study also showed that the reductase activity in F1B hamster liver was only affected by the dietary cholesterol. This resulted in a marginal reduction of the activity regardless the types of the fats (n-3 or n-6). However, the mRNA levels of the reductase were not affected by the dietary cholesterol, which may suggest a non-transcriptional regulation on the hepatic HMG-CoA reductase in F1B hamsters.

A recent study, in both rats and hamsters, showed that dietary PUFA (n-3, n-6) and cholesterol affected the cholesterol homeostasis differently in both serum and liver (Lin *et al.*, 2005), clearly indicative of species differences between rat and hamster in response

to the interactive effects of dietary n-3 PUFA and cholesterol. This may partly explain why our results differ from those reported by others.

4.2.3 Dietary Cholesterol and PUFAs on Sterol Regulatory Element Binding Protein-2

After we found that the dietary variation had no effect on the HMG-CoA reductase mRNA, we checked the expression of the transcription factor, SREBP-2, to see if it was regulated by dietary cholesterol and fats. Surprisingly, the levels of both the precursor and the mature form of the SREBP-2 in the livers of F1B hamsters were not regulated by the dietary fat and cholesterol.

According to the Brown and Goldstein model, the SREBPs are regulated by the cellular sterol and fatty acids levels through the SREBP-SCAP cascade (Brown and Goldstein, 1997e;Brown and Goldstein, 1999;Horton *et al.*, 2002). The proteolytic maturation of the protein stops when the system senses high levels of sterol in the cell. However, this fact could not explain why the dietary cholesterol only slightly suppressed the HMG-CoA reductase activity without any changes on the reductase mRNA.

Some studies have shown that the mature form of SREBP-2 can be slightly lower in mice fed a sardine oil-rich diet (Yahagi *et al.*, 1999) or by addition of arachidonic acid to rat hepatoma cells (Ou *et al.*, 2001). The mRNA of SREBP-2 was reduced in mouse liver by high fish oil diets. However, Jump et al. suggested that the PUFA only suppresses the mature form of SREBP-1 but not SREBP-2 (Jump, 2002;Jump, 2004). In our case, the SREBP-2 was not regulated by the dietary PUFA. In summary, it appears

that the SREBP-SCAP cascade, the major regulatory system for cholesterogenesis, was not affected by the dietary composition in F1B hamster liver.

4.2.4 Effect of Dietary Cholesterol and PUFAs on ACAT

It has been shown that the level of acyl-CoA cholesterol acyltransferase (ACAT) activity is mostly controlled by the intracellular availability of sterol (Chang *et al.*, 1995;Spady and Dietschy, 1988). Any physiological change that leads to a net increase in hepatic cholesterol induces the activity (Spady *et al.*, 1986), and similarly, any change decreasing the cholesterol in the liver suppresses the ACAT activity (Kovanen *et al.*, 1981). However, other factors like allosteric regulation (Chang and Chang, 1986;Tabas and Boykow, 1987) and post-translational regulation (Matsuda *et al.*, 1996;Wang *et al.*, 1996) have also been suggested to regulate ACAT.

Although it is generally believed that free cholesterol drives the activity of ACAT, fatty acids have also been shown to alter the activity (Rumsey *et al.*, 1995). Studies in rats fed polyunsaturated fat suggested that the animals had more PUFA in the fatty acyl chains of the microsomal phospholipids, which was associated with higher hepatic ACAT activity compared to the animals fed saturated tristearin (Mitropoulos *et al.*, 1980). However, a study in the F1B hamster showed that the hepatic ACAT activity was slightly suppressed by the dietary unsaturated fatty acids, compared to those fed saturated fat diets, and with no difference in the activity when animals were fed the linoleic acid or oleic acid diets (Lee and Carr, 2004). This study may indicate an opposite effect of PUFA on the ACAT activity in F1B hamster that is different from other animal models.

A study in normal hamster showed that, in the presence of 0.1% dietary cholesterol, menhaden oil enriched diets induced the highest rate of cholesterol esterification compared with animals fed the diets rich either in corn oil, or olive oil, or coconut oil. This suggested a positive interaction between the dietary fish oil and cholesterol on the ACAT activity (Jones *et al.*, 1990). A study in mice also suggested that the dietary PUFA (safflower oil) further increases hepatic ACAT activity in the presence of 1% dietary cholesterol.

Although these studies suggested that dietary PUFA induce ACAT activity and additional cholesterol further increases the activity, the suppressing effect of PUFA in F1B hamster may be regulated differently when the cholesterol is added to the diets. However, not many studies were focused on the interaction of the amount of the dietary fats, especially between the n-3 and n-6 fatty acids, and dietary cholesterol on the hepatic ACAT activity. In our study, the hepatic ACAT activity was induced by dietary cholesterol as we expected. Surprisingly, the amount of fat and types of fats (n-3, n-6) had no effect on the activity individually, but both factors have a positive interaction with dietary cholesterol. The high fat and fish oil rich diets induced the ACAT activity in F1B hamster when additional dietary cholesterol was added. However, without dietary cholesterol, increasing the amount of fat in both fish oil and MIX diets suppressed the ACAT activity compared to hamsters fed the low fat or even the control (chow) diets. This suggested that an opposite effect of dietary PUFA on ACAT activity in F1B hamster, dependent on the availability of dietary cholesterol. This fact implies that supplementation of diets with PUFA and cholesterol in hyperlipidemic subjects may not improve the serum lipid profile but rather it may induce higher ACAT activity, resulting

cholesterol ester accumulation in the liver and more VLDL-cholesterol ester secretion into the circulation and causing a severe hyperlipidaemia. Similar results were observed when normal hamsters were fed a diet with PUFAs and cholesterol (Lin *et al.*, 2005), and some clinical trails on familial hyperlipidemia patients also suggested that dietary n-3 fatty acids might not benefit the liprotein profile (Failor *et al.*, 1988;Hsu *et al.*, 2000).

The variation of the hepatic ACAT activity caused by the dietary differences therefore led us to expect a corresponding cholesterol ester accumulation in F1B hamster liver in a pattern close to the results from the ACAT activity assay. However, results from our previous study (de Silva et al., 2004) showed that the levels of cholesterol ester, free cholesterol, and total cholesterol were not reflective of the results of the ACAT assay. The in vivo ACAT activity is dependent on the availability of free cholesterol in cells, but the ACAT activity assay was performed *in vitro* with excess exogenous substrates provided in the form of a cholesterol-lipid emulsion. Thus the activity may not represent the ACAT activity that would be present *in* vivo with only the microsomal membrane cholesterol as the substrate. Using endogenous cholesterol as substrate in additional of radiolabeled cholesterol may be an alternative approach to estimate the real activity.

According to the hypothesis, ACAT activity is in part associated with VLDL secretion (Rudel *et al.*, 2001). It is possible that the elevation of ACAT activity by the high fat diets with cholesterol may subsequently increase the incorporation of the cholesterol ester into VLDL particles, which shifts cholesterol ester from the liver to the circulation. Although the results from the previous study showed a greater increase in the level of VLDL-cholesterol esters, the VLDL-CE from other diet groups didn't show a

pattern associated with ACAT activity. Therefore whether the ACAT activity is regulated by the dietary effect is still unclear.

In brief, we suggest that CYP7 and HMG-CoA reductase, and the corresponding transcription factor, SREBP-2, were not regulated by the variation of dietary polyunsaturated fats and cholesterol in F1B hamster. These facts surprised us due to the extensive literature that suggests these proteins are all regulated by dietary cholesterol and fats. These findings led us to examine whether the mechanisms that normally regulate SREBP, HMG-CoA Reductase and CYP7 were present and functional in the F1B hamster. In particular we focused on the effects of the lipid composition of the microsomal fractions from the F1B hamsters on these activities.

4.3 Dietary effects on Endoplasmic Reticulum Membrane Lipids

We observed that the levels of cholesterol in F1B hamster liver microsomes were affected by the amount and the type of fat in the diet. The high fat and fish oil diets appeared to facilitate more cholesterol incorporation into the microsomes. Surprisingly, the dietary cholesterol level showed only a minor impact on the microsomal cholesterol content when hamsters were fed fish oil and when the animals were fed different amounts of the MIX diet there was no effect on the microsomal cholesterol. We also found a positive interaction of dietary cholesterol and amount of dietary fat, with an increase in the microsomal cholesterol level when the animals were fed high fat diets with cholesterol.

The microsomes were rich in n-3 or n-6 fatty acids when the hamster was fed a fish oil or MIX diet, respectively. In addition, the diets had no effect on the levels of

microsomal monounsaturated fatty acids. However, when compared to the animals fed chow diet, the MUFAs were higher and PUFAs were lower in hamsters fed experimented diet. This indicated that under the influence of the diet, a portion of PUFAs were substituted by MUFAs. Moreover, the content of the major phospholipids (PE, PS, PC, SPH) were unaffected by the dietary variations. Thus the changes in the PUFA content of the microsomal membranes were limited to substitution of n-6 PUFA with n-3 PUFA.

4.4 Regression Analysis Between Microsomal Lipids and Enzyme Activity

The absence of a strong relationship between the dietary variations and the activities of microsome associated enzymes, other than ACAT, led us to perform a series of regression analyses between the microsomal cholesterol, n-3, n-6 fatty acids and the enzyme activities. We found the following:

1. A positive correlation between the ACAT activity and the content of microsomal cholesterol, and n-3 fatty acids respectively.
2. A positive correlation between the HMG-CoA reductase activity and the content of microsomal n-3 fatty acids.
3. A positive correlation between the content of microsomal cholesterol and n-3 fatty acids.

These results suggested that the activities of microsome associated enzymes, HMG-CoA reductase and ACAT directly depend on the changes of the microsomal environment in F1B hamster liver, especially the interactions between the cholesterol and n-3 fatty acids. However the specific mechanisms behind this were not clear.

4.4.1 Effects of Membrane Fluidity

An *in vitro* study suggested that unsaturated fatty acids are the preferred fatty acid substrate for ACAT (Rumsey *et al.*, 1995). *In vivo* studies in animals have shown that, compared with saturated fat diets, unsaturated fats increase the hepatic ACAT activity, probably by mediating changes in the microsomal fatty acid content and possibly the physical properties of the membrane environment where ACAT resides (Johnson *et al.*, 1983;Mathur *et al.*, 1983;Spector *et al.*, 1980). A study in rats showed that soybean lecithin supplementation decreased the liver microsomal cholesterol content, and increased the membrane fluidity. This resulted in a profound reduction of ACAT activity, a minor reduction on HMG-CoA reductase, and no significant effect on CYP7. Their results were closely relevant to ours and may further support the hypothesis that the activities of microsomal enzymes may be dependent on the membrane fluidity in the endoplasmic reticulum.

There was an absence of any correlation between the levels of cholesterol in the hamster liver microsomal fraction and the levels of either the precursor or mature forms of SREBP, the HMG CoA Reductase activity and the Cyp7 activity. However, it should be noted that the membranes of the F1B hamsters fed either the fish oil diet or the mix diet contained 30-45% of polyunsaturated fatty acids. There is a growing body of evidence showing that high levels of these polyunsaturated fatty acids in membranes can significantly alter the "fluidity" or microviscosity of membranes with concomitant effects on the activity of integral membrane proteins (Hashimoto *et al.*, 1999;Lutz *et al.*, 1999;Petrache *et al.*, 2001;Wassall *et al.*, 2004). The activity of HMG CoA Reductase in human fibroblast microsomes was shown to be attenuated by increases in order

(decreases in fluidity) of the microsomal membranes when the membrane order was altered either by changes in free cholesterol content of the membrane or by enrichment of the membranes with phospholipids containing saturated fatty acids (Davis and Poznansky, 1987). In this study we observed a positive correlation between HMG Co A Reductase and the content of n-3 PUFA in the microsomal membranes. It is possible that the ordering effect of cholesterol in the liver microsomes is being offset by the inclusion of polyunsaturated fatty acids, especially the high unsaturated n-3 PUFA from fish oil. If so, it is interesting that the n-3 PUFA are more effective than the n-6 PUFA as the n-3 PUFA contain more double bonds that the n-6 PUFA.

This being said, the fluidity effect would only be expected to impact the post-translational regulation of HMG CoA Reductase. It would be unlikely to account for the fact that the proteolytic cleavage of the SREBP precursor to the mature form is not affected by the different diets. The processing of SREBP is initiated by the conformational change of SCAP/SREBP complex when the cholesterol is low in the membrane and one would expect a correlation between microsomal cholesterol and the level of the mature form of SREBP. However, the levels of mature SREBP did not change in the hamster livers. The absence of any effects on SREBP processing might be accounted for by differential localization of the protein in the microsomal membrane.

4.4.2 Membrane Domains

Wassall et al. have proposed that inclusion of polyunsaturated fatty acids in membranes can result in substantial changes in the distribution of both the lipid and protein components of the membranes giving rise to cholesterol-rich raft domains and the

migration of proteins into cholesterol-poor bulk lipid (non-raft) (Wassall *et al.*, 2004) as illustrated in figure 4.1. This arises from a minimal interaction between cholesterol and phospholipids containing polyunsaturated fatty acids, and this is especially true for DHA, one of the two major n-3 PUFA in fish oil. It is therefore possible that the microsomes from the F1B hamsters fed the different diets contain cholesterol-rich domains from which much of the SREBP was excluded. The low cholesterol levels in the remaining bulk domains would then be insufficient to promote processing of the SREBP precursor into the mature form and it's subsequent translocation to the nucleus. This might account for the absence of any significant correlation between microsomal cholesterol levels and the levels of mature SREBP and concomitant enzyme activities.

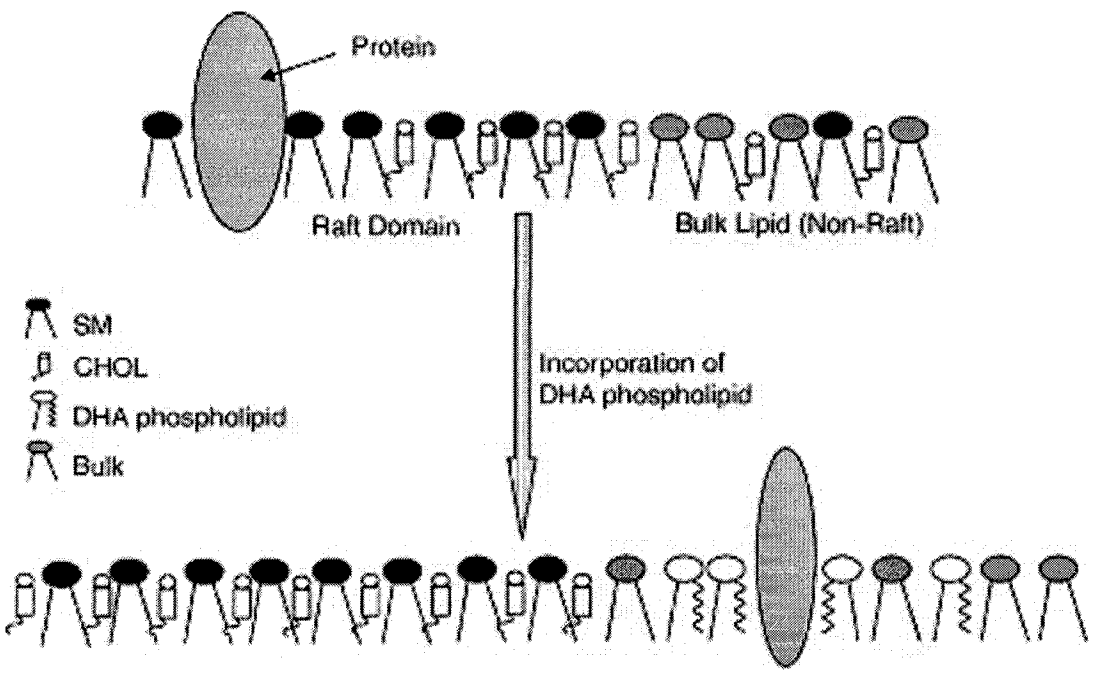
4.5 Summary

Another student in our lab had discovered that the effects of the amount of dietary fat, the types of dietary fat and the amount of dietary cholesterol on the lipoprotein profiles of the hypercholesterolemic F1B hamster were different from those reported in other species. The original objective of this work was to investigate the effects of these changes in dietary lipids in the diet of F1B hamsters on key enzymes involved in cholesterol homeostasis.

The impact of the different diets on microsomal enzymes is summarized in table 4.1. In our study, we observed that these microsomal enzymes are not especially responsive to the type of dietary polyunsaturated fat and cholesterol in the liver of F1B hamster. This raised questions about the functionality of the regulatory mechanisms that have been shown to account for the effects of dietary fat on cholesterol metabolism in the

Figure 4.1 Cartoon depiction of the effect of DHA-containing phospholipids on SM-/cholesterol-rich raft domains in the outer leaflet of the plasma membrane.

Wassall et al. proposed a cartoon illustration on how DHA may influence lipid raft size and stability. The incorporation of a DHA-containing phospholipid drives cholesterol into SM-/cholesterol-rich rafts while DHA accumulates in DHA-rich/cholesterol-poor domains, where the proteins reside, within the bulk lipid of the membrane. Steric incompatibility between sterol and PUFA is presumed to be responsible (Wassall et al. 2004).



(Wassall et al. 2004)

Table 4.1 Summary of the Results

Source of Variation	**Microsomal Lopids**		**Hepatic Enzyme Activities**				
Dietary effects	Cholesterol	n-3 FA	ACAT	CYP7	LDL-R	HMG-R	SREBP-2
High fat (A)	+			-	-		
Fish oil diet (B)	+	+			-		
Cholesterol (C)			+			-	
AXB							
AXC	+		+				
BXC			+				
Microsomal lipids							
Cholesterol		+	+				
n-3 fatty acid			+			+	

+ indicates a positive interaction
- indicates a negative interaction
Blank cell indicates no interaction

DSNI hamster and in other species. However, the numbers of animal in each diet group that were used in the "pilot" study was small and, in many of our analysis of enzyme activity or mRNA levels, n was only 2-3. Thus, the high variability may have precluded detection of significant effects of the changes in dietary fat and cholesterol.

Although we did not see major associations between diet and the activity of HMG-CoA reductase, CYP7, and SREBP-2 maturation was also not affected by diet, we were able to explore the relationships between the lipid content of the microaomal fractions and cholesterol regulations. We did regression analysis of our data and observed positive interactions between the microsomal n-3 fatty acids and cholesterol content in the microsomal membranes and the activities of HMG-CoA reductase and ACAT. We suggest that the activity of the enzymes may affected by the changes in the physical properties of the membrane induced by changes in dietary fat. There is also a possibility that the increased levels of phospholipids containing n-3 PUFA in the endoplasmic reticulum membrane may have led to changes in the lateral distribution of membrane lipids that result in the formation of cholesterol-rich domains (rafts) and cholesterol-poor domains. The key enzymes in cholesterol metabolism would likely be partitioned into the cholesterol-poor domains and thus be unresponsive to cholesterol content in the membranes.

4.6 Future Study

In order to find out whether the existence of microdomains plays a role in the regulation of the enzyme activity, we could determine if the two domains, the SM-/cholesterol-rich rafts as well as PUFA-rich/cholesterol-poor microdomains, exist in the

microsome using various techniques including Nuclear Magnetic Resonance Spectroscopy (NMR) or Atomic Force Microscopy (AFM). Also if there is evidence that these domains exist, the cholesterol-rich lipid rafts could be isolated and the partition of the enzymes and SREBP between the rafts and the bulk lipid domains could be examined.

It is also possible to account for the unusual effects of dietary lipids on the plasma lipoprotein levels in the F1B hamster was modulated by the assembly and secretion of VLDL by the liver. There is some suggestion that the type of fatty acids in the diet may affect the activity of the microsomal triglyceride transfer protein (MTTP). It would by interesting to determine if MTTP activity is regulated by the diet or the microsomal environment in the F1B hamster liver to determine if this might account for the levels of hepatic cholesterol ester and plasma VLDL-cholesterol ester.

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