1 Introduction

Cholesterol, fatty acids, phospholipids and triglycerides are important lipids in the body (Luskey 1988, Semenkovich 1997). Lipids are a major source of energy (triglycerides) and have many other functions such as the structure of membrane (phospholipids) and hormone synthesis (prostaglandins and steroid hormones). However, imbalances of lipid metabolism can lead to some of the major clinical problems such as atherosclerosis and obesity (Semenkovich 1997, Stamler et al. 1986). Atherosclerosis is the main cause of coronary heart disease (CHD), which is the leading cause of mortality in the western countries (Glass and Witztum 2001, Grundy 1994). Atherosclerotic vascular disease develops from complex multifactorial processes that contribute to the deposition and accumulation of cholesterol in focal areas of the arterial wall (see Figure 1.1). Elevated or modified blood levels of total cholesterol, particularly low density lipoprotein (LDL) cholesterol, free radicals caused by cigarette smoking, hypertension, elevated plasma homocysteine level, infectious microorganisms, and combinations of the above are risk factors for CHD (Grundy 1996). There is substantial evidence that lowering total and LDL-cholesterol levels reduce the incidence of CHD and coronary death (Grundy 1996). Many primary and secondary prevention trials have proven the benefits of cholesterol-lowering (Grundy et al. 1999). Aside from cholesterol, elevated levels of plasma triglyceride have been associated with an increased risk of cardiovascular disease (Austin 1991). A meta analysis revealed that increased triglyceride level is a risk factor for cardiovascular disease independent of high density lipoprotein (HDL) cholesterol level (Austin et al. 1998). Studies in both human and animal models have shown that triglyceride-rich lipoproteins such as intermediate density lipoprotein (IDL) and very low density lipoprotein (VLDL) are related to the extent and severity of atherosclerosis (Krauss 1998). Lowering triglyceride levels may reduce the risk of CHD (Ericsson et al. 1996).

One of the strategies to lower blood lipids is lifestyle intervention such as diet modifications and controlling body weight (Kris-Etherton et al. 1997). In October 1999, the FDA approved a claim stating that 25 grams of soy protein a day, as a part of diet low in saturated fat and cholesterol may reduce the risk of heart disease. Soy protein or lupin has been shown to reduce plasma lipid levels in studies with human and animal models (Bakhit et al. 1994, Ni et al. 1998, Anderson et al. 1995, Wang et al. 1998, Potter et al. 1998, Sirtori et al. 2004). Recent research shows that the effects of soy protein include decreases in plasma total cholesterol and LDL-cholesterol concentrations with an increase in HDL-cholesterol

levels in different model animals and hyperlipidemic patients when compared to an animal protein diet mainly casein (Anderson et al. 1995, Wang et al. 1998, Sirtori and Lovati 2001, Madani et al. 1998, Potter 1996). The hypocholesterolemic effect is directly correlated to the patient's cholesterolemia.

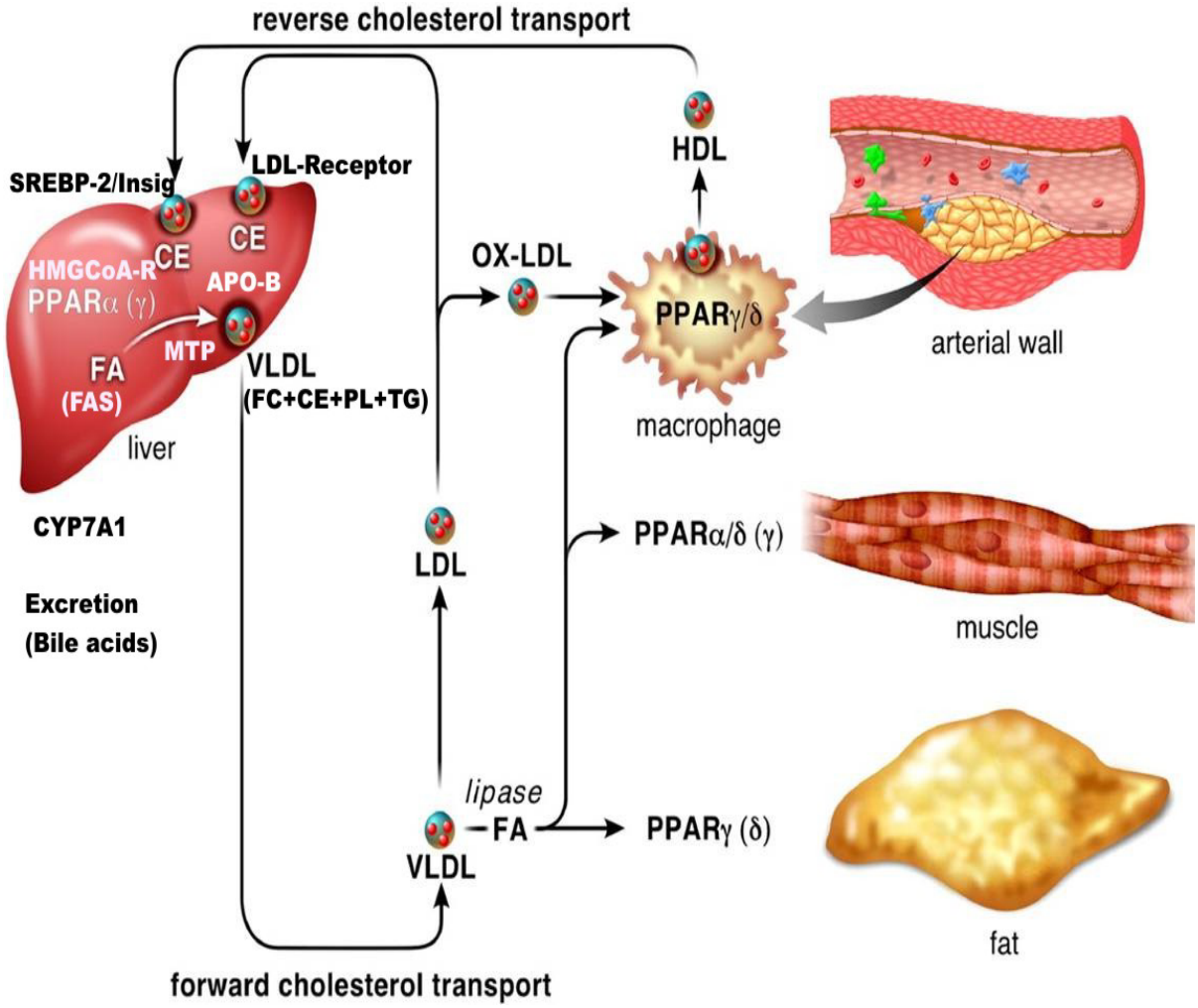

Figure 1.1: Cholesterol transport cycle. The liver secretes cholesterol in the form of VLDL. VLDL can subsequently be converted into LDL. In humans, LDL particles contain most of the cholesterol and the oxidized form of LDL can deposit in the artery wall (www.Google.com). VLDL, very low density lipoprotein; LDL, low density lipoprotein; ox-LDL, oxidized LDL; HDL, high density lipoprotein Apo-B, apolipoprotein-B; HMG CoA R, 3-hydroxy 3-methylglutaryl coenzyme A reductase; PPAR α (γ), peroxisome proliferators activated receptor alpha (gamma); FAS, fatty acid synthase; MTP, microsomal triglyceride transfer protein; Cyp7A1, cholesterol 7 alpha hydroxylase; SREBP, sterol regulatory element binding protein; Insig, insulin induced gene; FA, fatty acids; PL, phospholipids; TG, triglycerides; FC free cholesterol; CE, cholesterol ester.

There are several components of soybeans that may contribute to the lipid lowering properties of soy. Components such as soy protein, amino acids, peptides, isoflavones, saponins, phytic acid, fibers and protease inhibitors have been implicated in the hypolipidemic effect of soy protein preparations (Anderson et al. 1995, Wang et al. 1998, Potter et al. 1998). The major isoflavones (phytoestrogen) in the soybeans are genistein and daidzein. The hypocholesterolemic effect of isoflavones was noted in mice (Kirk et al. 1998), hamsters (Balmir et al. 1996), non human primates (Anthony et al. 1997) and humans (Wang et al. 1998, Carroll and Kurowska 1995, Gardner et al. 2001, Damasceno et al. 2001). Lichtenstein et al. (2002) reported that ingestion of ethanol extract rich in isoflavones increases the abundance of hepatic mRNA for cholesterol 7α-hydroxylase (CYP7A1) and LDL receptors in rats, which play important roles in cholesterol catabolism. However, other studies demonstrated that soy protein rather than its isoflavones contribute to the lipid lowering properties of soy (Claudia et al. 1998, Adams et al. 2002, Jenkins et al. 2002, Lichtenstein et al. 2002). Moreover, soy protein enhances the expression of the LDL receptors in hypercholesterolemic type II diabetic patients (Lichtenstein 1998, Wang et al. 1998, Potter 1995), animals (Iritani et al. 1996, Iritani et al. 1986, Wright & Salter 1998) and cultured human hepatoma cells. But these effects of soy protein were strongly related with the subject's initial serum cholesterol concentrations. It is also shown that the methionine content of the dietary protein is positively related with serum total cholesterol concentration in rats and atherosclerosis like alterations in the aorta of rats (Sautier et al. 1983, Antony et al. 1998). It is also reported that dietary supplementation of L-arginine prevents intimal thickening in the coronary arteries of hypercholesterolemic rabbits (Forsythe 1995). In vitro studies have clearly indicated that specific protein fractions of soybean, in particular the alpha' subunit of the 7S globulin and thereof peptides can directly activate LDL receptors in liver cells (Lovati et al. 2000). All these studies showed partly different effects and the underlying mechanism of this lipid lowering effect is not fully understood yet.

Therefore one aim of our first experiment was to investigate the mechanism of soy protein isolate on lipid metabolism, to minimize interference of isoflavones the soy protein isolate used for this study was additionally ethanol-washed. The results indicate that ethanol extracted soy protein also has positive effects on lipid metabolism.

In general, animal proteins are considered to be hypercholesterolemic when compared with plant proteins. But the number of proteins examined in this connection, is so far very limited. Most of the studies are based on casein as representative of animal proteins and soy

protein as representative of plant proteins (Ni et al. 1998, Damasceno et al. 2001). Besides casein, animal proteins such as those from beef, pork, poultry, or fish protein play an important role in human nutrition worldwide. Therefore, we planned a second experiment to investigate the effects of meat proteins isolated from pork, beef, and turkey, and fish protein isolated from Alaska pollack fillets and compared their effects on the lipid metabolism with casein and ethanol washed soy protein isolate. Casein served as reference protein of animal origin, and soy protein isolate, which is known to have hypocholesterolemic action when compared with casein, served as reference protein of plant origin. To study whether dietary proteins isolated from beef, pork, turkey and fish influence the metabolism of cholesterol or triacylglycerols, we determined the concentrations of cholesterol and triacylglycerols in plasma, lipoproteins and liver. In the liver, moreover, we determined the ratio between esterified and free cholesterol to investigate whether esterification of the free cholesterol could be influenced by these animal proteins.

In a third experiment, we decided to investigate alternative legume proteins isolated from peas and sweet lupin seeds. Moreover, we intended to verify the results obtained after feeding fish protein in the second experiment because until now there is little knowledge available about the effects of fish protein on lipid metabolism. Bergeron and Jacques (1989) demonstrated that the serum cholesterol level of rabbits fed fish protein was intermediate and not different from that of rabbits fed casein or soy protein. Iritani et al. (1985) reported that feeding fish protein to rats had a hypocholesterolemic effect equivalent to that of soy protein rather than casein. Thus fish protein has been shown to induce variable effects on serum cholesterol level compared with casein and soy protein (Jacques et al. 1995).

As it has been suggested that protein induced alterations of the lipid metabolism could be mediated by certain amino acids such as methionine, lysine or arginine (Sugiyama et al. 1996, Sugiyama et al. 1997, Gudbrandsen et al. 2005), we measured the concentrations of amino acid in the diets and in plasma. The synthesis of cholesterol and fatty acids begins with a common precursor, acetyl CoA, which originates from the catabolism of carbohydrates, proteins, and lipids. Acetyl CoA is converted to cholesterol in a pathway involving at least 23 enzymes. Acetyl CoA is also polymerized to form fatty acids in a pathway involving up to 12 enzymatic steps. Therefore, we planned to analyze lipid metabolism at the level of activities of enzymes like glucose 6-phosphate dehydrogenase (G6PDH), fatty acid synthase (FAS), and microsomal triglyceride transfer protein (MTP). Moreover, we intended to analyze the mRNA concentrations of various proteins involved in lipid metabolism like 3-hydroxy-3-

methylglutaryl-CoA reductase (HMG CoA R), apolipoprotein-B (APO-B), LDL receptor, delta 6-desaturase, FAS, CYP7A1, acyl CoA oxidase (ACO), and cytochrome 450 4A1 (CYP4A1). Since the effects of fish protein are less investigated we intended to apply the cDNA macro arrays which allow insight in the expression of more than 1000 proteins/genes at once. We decided to evaluate the gene expression profile of the liver because this tissue is the major site of lipid metabolism.

In the regulation of lipid metabolism several transcription factors like peroxisome proliferator-activated receptor α (PPAR-α) and sterol regulatory element binding proteins (SREBPs) are involved. Therefore we analyzed the expression of PPAR-α and SREBPs.

PPARs are nuclear proteins that belong to the super family of nuclear hormone receptors. They mediate the effects of small lipophilic compounds such as long chain fatty acids and their derivatives on transcription of genes commonly called PPAR target genes. Of the three PPAR types (PPAR-α, PPAR-β and PPAR-γ) known to date, PPAR-α has been best characterized. PPAR-α is involved in the regulation of peroxisomal β-oxidation (ACO, Thiolase B), mitochondrial ω-oxidation (CYP4A1, 4A6-Z), mitochondrial β-oxidation (CPT-I, CPT-II, MCAD). Thus, PPAR-α serves as a master regulator of fatty acid catabolism (Mandard et al. 2004, Dreyer et al. 1992).

SREBPs are a family of three proteins (SREBP-1a, SREBP-1c and SREBP-2) which control lipid metabolism in the liver and other organs (Horton et al. 2002). SREBPs are intrinsic membrane proteins of the endoplasmic reticulum (ER). SREBP-1a and SREBP-1c both activate genes involved in the synthesis of fatty acids and their incorporation into triglycerides and phospholipids. SREBP-2 preferentially activates genes for cholesterol synthesis and the LDL receptor (Horton et al. 1998). In liver SREBP-1c and SREBP-2 are the predominant isoforms. SREBP-1a is present in lesser amounts (Shimomura et al. 1997).

We also analyzed Insulin Induced Gene (Insig) proteins, which are recently identified ER proteins (Insig-1 and Insig-2). The gated movement of SREBPs from ER to the Golgi complex is the central event in lipid homeostasis in animal cells, which is controlled by Insig proteins (Horton et al. 2002). The combined actions of Insig-1 and Insig-2 permit feedback regulation of cholesterol synthesis over a wide range of sterol concentrations (Yang et al. 2002). The SREBP precursor protein is anchored in the membranes of the ER and nuclear enveloped by two membrane-spanning helices (Figure 1.2) (Hua et al. 1995).

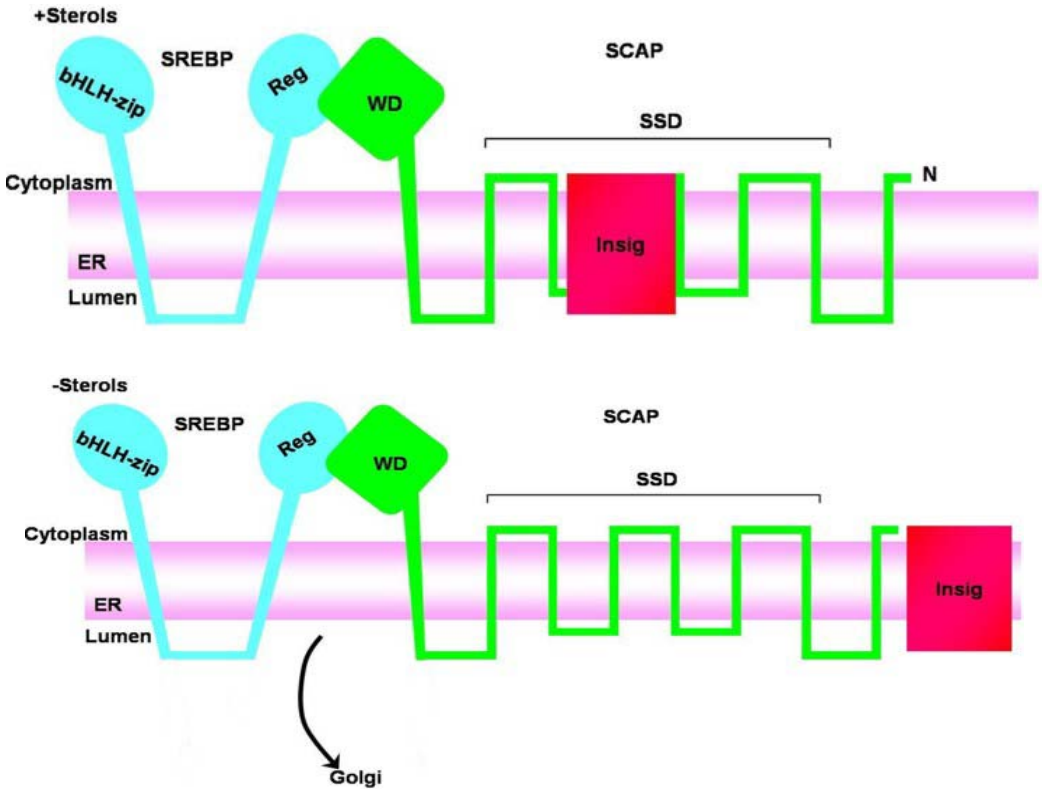

Figure 1.2: Sterol-regulated interaction between Insig and SCAP. In the presence of sterols, the SREBP-SCAP complex remains in the endoplasmic reticulum (ER). Retention in the ER requires interaction between the sterol sensing domain (SSD) of SCAP and Insig-1 or 2. In the absence of sterols, SCAP and the Insig proteins do not interact. The SREBP-SCAP complex is then free to travel to the Golgi apparatus to be processed (Rawson 2003). bHLH-zip, basic helix-loop-helix leucine zipper; Reg, regulatory; WD, aspartate-tryptophan motif; SCAP , SREBP cleavage activating protein; Insig, Insulin induced gene; N, amino terminal.

As shown in Figure 1.2, when cellular cholesterol levels are high, Insig proteins bind and trap proteolytic activation in the Golgi complex and therefore the level of SREBPs in the nucleus drops resulting lower expression of genes involved in lipid metabolism. When the cellular demand for the cholesterol rises, the SREBP-SCAP (SREBP-cleavage-activating protein) complex leaves the ER and travels to the Golgi apparatus where the SREBP-SCAP complex cleavage occurs by two proteases, site 1 protease and site two protease. This releases the transcriptionally active SREBPs, which can then travel to the nucleus where it can activate the transcription of target genes (Rawson 2003).