# 4 Discussion

It has been established that dietary proteins influence lipid metabolism in men and animals (Anderson et al. 1995, Sirtori et al. 1998, Sirtori & Lovati 2001). Coronary heart disease (CHD) mortality and morbidity in Asian countries are substantially lower than in western countries (Robertson et al. 1997). Adlercreutz (1990) has suggested that this may be due to the considerably higher intake of soy protein in Asian countries. Previous studies, replacing dietary animal protein with intact soy protein, have shown significant improvements in CHD risk factors, particularly total cholesterol, LDL-cholesterol and triglycerides in humans and laboratory animals (Anderson et al. 1995, Carroll 1991, Carroll and Kurowska 1995). It has been reported many times that soy protein has hypocholesterolemic (Anderson et al. 1995, Iritani et al. 1996, Sugiyama et al. 1996) and hypotriglyceridemic action (Tovar et al. 2002, Ascencio et al. 2004, Tovar et al. 2005) in laboratory animals, and humans when compared to casein. However, the components of soy that may contribute to the lipid lowering properties have not been well characterized. Several studies have been made to elicit possible mechanisms by which soy protein isolate may act on lipid metabolism. Based on studies with cell cultures, animals and humans, suggested mechanisms of soy protein include increases of low-density lipoprotein (LDL) receptor expression and activity (Lovati et al. 1987, Sirtori et al. 1984, Lovati et al. 1991, Manzoni et al. 1998, Lovati et al. 2000), down regulation of sterol regulatory element-binding protein-1 (SREBP-1), a transcriptional factor that is primarily responsible for the regulation of genes involved in fatty acid biosynthesis (Tovar et al. 2002, Ascencio et al. 2004), and increases in the synthesis and faecal excretion of bile acids (Tachibana et al. 2005). Tachibana et al. (2005) have found that soy protein isolate significantly up-regulates the expression of genes related to steroid catabolism that may result in a reduction of the serum cholesterol level. Other experiments with cultured hepatocytes have indicated that certain peptide components of soy protein stimulate expression of LDL receptors (Lovati et al. 1992, 1996). However, the results of a study in LDL receptor null mice did not support widely suggested LDL receptor mediated mechanism (Adams et al. 2002), in which soy protein isolate lowers the LDL and VLDL cholesterol concentrations and inhibited atherosclerosis despite the absence of LDL receptor in the mice. The LDL receptor is one of a number of genes regulated by the transcription factor SREBP-2. SREBP-2 is critical for the regulation of intracellular sterol homeostasis and besides regulation of LDL receptor expression SREBP-2 is primarily responsible for the regulation of genes involved in cholesterol biosynthesis such as 3-hydroxy 3-methylglutaryl CoA (HMG CoA) reductase

(Hua et al. 1993, Vallett et al. 1996, Horton et al. 2002). Although, recent evidence exist that rats fed a soy protein diet have reduced SREBP-1 expression (Tovar et al. 2002, Ascencio et al. 2004), however, the expression of SREBP-2 and its sterol regulatory element-related genes that may also play a role in the hypolipidemic effects of soy protein isolate have not yet been investigated.

Therefore, one aim of our first experiment was to investigate the effects and mechanism of soy protein on the cholesterol metabolism. To minimize possible effects of soy protein-associated isoflavones that may partly be responsible for cholesterol reduction by soybean protein preparations, the soy protein isolate used in our studies was additionally ethanol-washed. To mimic western diets, which are commonly rich in saturated fats and cholesterol, we chose lard as type of dietary fat and supplemented the diet with cholesterol (0.5 g/kg). In spite of removal of most of isoflavones by ethanol extraction, the soy protein diet was able to show effects on cholesterol and triglyceride metabolism.

Besides casein, animal proteins such as those from beef, pork, poultry and fish play an important role in human nutrition worldwide. Yet less is known about their effects on the lipid metabolism. Therefore, we planned a second experiment to investigate the effects of proteins isolated from beef, pork, turkey meat and fish protein isolated from Alaska pollack fillets on lipid metabolism and compared their effects with casein and soy protein. Casein served as reference protein of animal origin; and soy protein served as reference protein of plant origin.

In a third experiment, we planned to investigate the effect of other easily available and commonly used plant proteins isolated from peas (used in infant formula food) and sweet lupin seeds. Moreover, we intended to affirm 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.

As parameters of lipid metabolism, concentrations of cholesterol and triglycerides in plasma, lipoproteins, and liver, faecal excretion of bile acids, activities and concentrations of selected enzymes and hepatic expression of genes encoding proteins involved in lipid homeostasis were determined. In all three studies we used rats as model animal, a model which is used in many investigations of the dietary proteins (Iritani et al. 1996, Ascenio et al. 2004, Gudbrandsen et al. 2005). Growing rats were used, as we expected that the effects of the dietary treatments would be greater than in adults. We evaluated the gene expression profile and enzyme activity of the liver because this tissue is the major site of lipid metabolism. To avoid the interference of chylomicrons and postprondial rise of lipids, we

sacrificed the animals of the first two studies after overnight fast. However, under these conditions a lot of regulating proteins were probably down regulated (Hortan et al. 1998, Shimano et al. 1999) and the effects of proteins are indistinguishable. Therefore, in the third study we sacrificed animals in the postprandial state. The differences in the observations of the three experiments can be explained by different amount of food ingested during different experiments.

We used a relatively short experimental period as recent studies have shown that effects of dietary proteins on the lipid metabolism of rats become manifest within a short period, usually within two weeks or even earlier (Sugiyama et al. 1996, Sugiyama et al. 1997, Iritani et al. 1986, Ascencio et al. 2004). Most of the effects of soy protein on lipid metabolism observed in this study are in accord with those reported by other investigators (Madani et al. 1998, Madani et al. 2003, Tover et al. 2002, Ascencio et al. 2004). The fact that rats fed the soy protein diet exerted markedly alterations of their lipid metabolism confirms that the feeding period was long enough to adequately study effects of dietary proteins on the lipid metabolism.

### 4.1 Body and Liver Weight

Although the rats were fed a restrictive diet in order to exclude secondary effects which might result from different feed intakes, in the first two studies casein fed rats grew at the faster rate, whereas rats fed soy protein grew at slower rate. These results are in agreement with Tovar et al. (2002), Iritani et al. (1996), and Zhang and Beynen (1993). Tovar et al. (2002) observed that in spite of similar food intake the casein fed rats grew at the faster rate than soy protein fed rats. The growth retardation by the soy protein diet may be due to the lower concentration of lysine in the soy protein compared to casein, because lysine has been reported to be an important factor for growth. In the third experiment, there was no difference observed in the body weight gain between rats fed casein and soy protein, but in the third experiment additional DL-methionine was supplied to the soy protein diet. Therefore, the lower methionine concentration in the soy protein might be at least in part responsible for the slower growth rate of rats fed this protein.

In third experiment, rats fed lupin protein grew at the slower rate, compared to rats fed casein or soy protein. Few of the rats fed lupin protein containing diet showed health problems from the second week of feeding (skin hair fall etc.) and this group rats did not

consume all the food provided daily, which may explain the lower body weight gain. The health problems observed by feeding lupin protein diet might be because of higher content of plant alkaloid or antinutritional facors (like tannins and protease inhibitors etc.) found in the plant proteins, although the protein was recommended for nutritional use by the supplier.

In first two experiments, the relative liver weight of rats fed soy protein was significantly lower compared to rats fed casein. In third experiment, the relative liver weight was lower of rats fed pea or lupin than rats fed casein. These results are in agreement with many previous studies. Sugiyama et al. (1996) reported significantly lower relative liver weight with soy protein than with casein and fish protein diet. Iritani et al. (1986) also reported that the liver weight relative to body weight was lower in rats fed gluten or soybean protein than in those given casein or fish protein . Terpstra et al. (1983) and Peluso et al. (2000) also reported substitution of soy protein for casein reduced the liver weight. Zhang and Beynen (1993) reported soybean protein induced significantly lower liver weight than did either casein or cod meal.

## 4.2 Cholesterol Concentration

In all three experiments, rats fed soy protein and in experiment 3 rats fed pea and lupin protein had much lower cholesterol concentration in liver and VLDL compared to rats fed casein. In the first two experiments the ratio of LDL-/HDL-cholesterol was significantly lower in rats fed soy protein compared to rats fed casein. These results are in agreement with Damasceno et al. 2001, who reported that soy protein isolate in comparison with casein, promoted a decrease of cholesterol and triglyceride content of atherogenic lipoproteins. The lower cholesterol concentrations by soy protein diet were accompanied by a lower gene expression of SREBP-2. SREBPs are important transcription factors which belong to the basic helix-loop-helix-leucine zipper family and bind in its activated form to the sterol regulatory element in the promoter or enhancer regions of genes involved in cholesterol or fatty acid synthesis (Hua et al. 1993, Shimano et al. 1999). SREBP transcription factors are synthesized as inactive precursors bound to the endoplasmic reticulum membranes. Activation of these membrane-bound transcription factors involve a two-step proteolytic cascade through which the SREBP molecule is released from the membrane and obtains its mature form as a transcription factor which enters in the nucleus. Although, we did not measure the mature fraction of SREBP in nucleus because there was not enough sample material available from each animal, several additional parameters indicate an actually lower activity level of SREBP

in rats fed soy protein compared to rats fed casein. Parameters indicating a diminished activity level of SREBP-2 were a lower gene expression of HMG-CoA reductase and of LDL-receptor, both are downstream genes of SERBP-2, as well as diminished concentrations of cholesterol in liver.

Plasma cholesterol concentration did not differ between rats fed casein and soy protein. These observations are in agreement with Tovar et al. (2002), Madani et al. (2003), Sugiyama et al. (1996), and Ni et al. (1998), who reported no effect on serum total cholesterol concentration in soy protein isolate fed rats and mice, compared with those fed casein. Lucas et al. (2001) reported that ethanol extracted soy protein isolate does not modulate serum cholesterol concentration in ovarian hormone-deficient Golden Syrian hamsters. Van Raaij et al. (1981, 1982) assessed the effect of casein, soy protein isolate and soy protein concentrates on serum cholesterol levels in young and middle aged healthy humans. They reported that neither soy protein preparation had a significant effect on blood cholesterol levels compared with casein. But these observations are in contrast to a number of animal (Potter 1996, Carroll and Kurowska 1995) and human studies (Anderson et al. 1995) in which soy protein isolate or other soy bean products exerted hypocholesterolemic actions, compared to casein based diets. This effect is somewhat variable but is generally greater in hypercholesterolemic subjects than in normocholesterolemic subjects.

There was no effect of soy protein on the LDL cholesterol concentration in our studies, this is in contrast to a series of previously published results that show a distinct reduction of plasma and/or LDL cholesterol after administration of soy protein in man and animals (Bakhit et al. 1994, Carroll 1991, Carroll & Kurowska 1995). We suggest that lower mRNA concentrations of LDL receptor in rats fed soy protein isolate may explain the finding that plasma LDL concentration remained unchanged although liver cholesterol concentration was markedly decreased by dietary soy protein compared to casein. The LDL receptor is a major regulator of circulating LDL cholesterol (Meddings et al. 1986). Decreased removal of LDL from the circulation due to a diminished LDL receptor expression may increase plasma or LDL cholesterol concentration albeit down regulated cholesterol synthesis by soy protein isolate. LDL receptor expression lowering effect of the soy protein is in agreement with Ni et al. (1999), who reported that the isoflavone-intact soybean protein, but not the alcohol-extracted soybean protein enhanced hepatic LDL receptor mRNA in exogenously hypercholesterolemic rats. Additionally, in most of the studies cited in the literature the animals were fed soy protein, which was not characterized regarding its isoflavones.

Therefore, results from those experiments cannot actually be compared with the results from our experiments.

Our experiments are moreover the first showing that modulation of SREBP-2 by isoflavone-poor soy protein isolate was not due to an altered expression of Insig 1 and 2 (Shukla et al. 2006 b). Insigs are protein regulators that prevent movement of the SREBP/SCAP complex from the endoplasmic reticulum to the Golgi, thus blocking proteolytic cleavage and transcriptional activation of SREBP (Yang et al. 2002, Yabe et al. 2002). The reduced mRNA and protein concentrations of apo-B100 and apo-B48 could also be a consequence of altered intracellular cholesterol content. It has been shown that the secretion rate of lipoprotein cholesterol from the liver in rats fed a soybean protein diet is significantly lower than in rats fed a casein diet (Sugano et al. 1982, Pfeuffer and Barth 1986).

In our studies the main effects of the fish protein on cholesterol metabolism compared to casein were an increase of liver cholesterol concentration, a reduction of the HDL cholesterol concentrations, along with a stimulated gene expression of scavenger receptor class B type I (SR-B1), SREBP-2, and FAS. The higher relative mRNA concentrations of SREBP-2 in rats fed fish protein suggest that the cholesterol synthesis might possibly be stimulated by the fish protein which in turn could be responsible for the observed accumulation of cholesterol in the liver. This assumption is supported by the findings of Wergedahl et al. (2004) who observed a higher activity of HMG-CoA reductase in rats fed fish protein compared to those fed casein, although this effect was only observed in genetically hyperlipidemic obese Zucker rats but not in normal Wistar rats. In our experiments, besides a slight increase of HMG-CoA synthase expression, gene expression of LDL receptor was also slightly increased in rats fed fish protein. Both are target genes of SREBP-2 and indicate an activation of this transcription factor by fish protein feeding. Fish protein fed rats had significantly higher liver cholesterol and cholesteryl esters concentration compared to casein or soy protein fed rats. The increase of cholesteryl esters in the liver of rats fed fish protein support the fact that enlarged amounts of cellular cholesterol are normally associated with a higher concentration of esterified cholesterol (Field et al. 1987). Since the hepatic cholesterol homeostasis is achieved by a balance of biosynthesis, storage, catabolism, and export processes that influence cholesterol excretion could contribute to the observed cholesterol accumulation in livers of fish protein-fed rats. Abundant cholesterol is normally eliminated from liver mainly via bile acids and CYP7A1 is the key enzyme of synthesis of bile acids from cholesterol (Vlahecevic et al. 1999). However, since expressin of CYP7A1 in

the liver and the amounts of bile acids excreted via faeces, was higher in the fish protein fed rats compared with casein fed rats, it is suggested that the cholesterol accumulation in livers of fish protein-fed rats was not due to the reduced excretion of the cholesterol via bile acids.

Despite the observed cholesterol accumulation in livers of rats fed fish protein the concentration of cholesterol in plasma was not increased compared to rats fed casein. By measuring the different density lipoprotein fractions in plasma from rats of both experiments we found a lower cholesterol concentration in the HDL fraction in those fed fish protein compared to casein. The cut off chosen for lipoprotein was typical for humans, but also matched that used in previous rat studies (Sparks et al. 1998, Giudetti et al. 2003, Sirtori et al. 2004). As rat lipoproteins have a different density compared to human lipoproteins. This means the 1.006 < δ> 1.063 lipoprotein fraction contained besides LDL also intermediate density lipoproteins (IDL) and some HDL lipoproteins and this is possibly the reason for the inconsistent effects of fish protein on the lipoprotein fraction in both experiments. A major regulator of circulating LDL cholesterol is the LDL receptor (Meddings et al. 1986). The observed higher expression of LDL receptor in livers of rats fed fish protein could have possibly contributed to an increased removal of LDL from the circulation, thereby preventing a distinct LDL accumulation in plasma albeit an increased expression of SREBP-2.

The HDL-cholesterol lowering effect observed with fish protein feeding is in agreement with the recent findings in hyperlipidemic obese zucker rats and in hamsters that were fed fish protein from salmon (Wergedahl et al. 2004, Tsai and Huang 1999), and in humans fed fish diet (Li et al. 2004). HDL is the principle vehicle for removal of surplus cholesterol from the peripheral tissues for disposal in the liver. Several genes are involved in HDL metabolism like Apo-AI, a structural component of HDL, whereas the esterification of cholesterol by lecithin-cholesterol acyl transferase (LCAT) is critical for optimal cholesterol uptake and maturation of HDL (Genest et al. 1999) and SR-BI, otherwise known as HDL receptor, responsible for the selective uptake of HDL and unloading of HDL cholesterol in the liver (Acton et al. 1996). cDNA array results of Apo-AI and LCAT were not different between rats fed casein and fish protein. Although, the observed reduction in HDL-cholesterol concentration in rats fed fish protein could possibly be related to an increased expression of SR-BI. Further investigations will be necessary to clarify the mechanisms.

This study demonstrates that proteins from beef, pork and turkey did not alter the concentrations of cholesterol in plasma, lipoproteins, and liver and the ratio of esterified to free cholesterol in the liver compared with casein. This is consistent with previous work

showing that proteins from beef and chicken meat and also egg albumin and ovalbumin had no influence on cholesterol concentration in liver and plasma compared with casein (Lapre et al. 1989). Scott et al. (1994) showed that a diet with lean beef or chicken and fish had similar effects on serum lipoproteins of men with borderline hypercholesterolemia.

Since dietary proteins had to be treated in this way for experimental reasons, we cannot exclude the possibility that they were denaturized by the isolation procedure and that functional peptides in the proteins were changed. Therefore, we cannot absolutely exclude the possibility that the same proteins could have induced different effects on the lipid metabolism if they would have been prepared in another way such as under cooking condition.

### 4.3 Triglyceride Concentration

Soy protein lowered Triglyceride concentrations in liver, plasma, VLDL, LDL and HDL compared to casein. These results are in agreement with number of previously reported studies. Horigome and Cho (1992) have shown that soybean protein compared with casein results in lower plasma triglyceride levels. Sugiyama et al. (1996) and Terpstra et al. (1983) reported that the hepatic triglyceride concentration of soybean fed rats was significantly lower than that of casein and fish protein fed rats. Lucas et al. (2001) reported that ethanol extracted soy protein isolate lowered serum triglyceride concentration in Golden Syrian hamsters. In our study, SREBP-1c and FAS expressions were down regulated by soy protein compared to casein in experiment 1. Lower activities of FAS and G6DPH observed in experiment 2 show that soy protein lowers triglyceride synthesis. These results confirm the findings from previous studies that have found a decreased expression of SREBP-1, and FAS in rats fed soy protein compared to rats fed casein (Ascencio et al. 2004, Tovar et al. 2002). Ascencio et al. (2004) suggested that soy protein regulates SREBP-1 expression by modulating serum insulin concentration. Iritani et al. (1986) reported that the activity of lipogenic enzymes (G6PDH, malic enzyme, acyl CoA carboxylase, and FAS) in the whole liver of rats fed gluten or soybean protein were reduced to half the levels found in the rats fish protein or casein. It is well established that plasma triglycerides are supplied by the triglyceride-rich lipoproteins VLDL, in the fasted state, and by VLDL and chylomicrons, in the non-fasted state (Tso et al. 1984). Rat used in the first two experiments were food deprived for 12 h but the rats used in third experiment were non-fasted before killing because observations from our laboratory and a study of Shimano et al. (1999) have shown that food deprivation a few hours before killing led to a significant down regulation of genes involved in lipid metabolism. The VLDL

fractions obtained from plasma of our third experiment rats were contaminated with chylomicrons from intestine. In plasma, the concentrations of both apolipoproteins B-100 and the B-48 were reduced in rats fed soy protein compared to rats fed casein. This demonstrates that the soy protein lowered VLDL particle number. Similar findings were reported in rats and mice (Madani et al. 2003, and Nagata et al. 1981) and in soy-treated postmenausal women (Vigna et al. 2000). This might, in part result from diminished VLDL production and in part result from enhanced VLDL uptake. Madani et al. (2003) have shown that lower plasma VLDL particle number and plasma triglyceride concentrations in rats fed soybean protein compared with casein resulted from an enhanced VLDL uptake in the liver. In our study, the lower MTP activity as well as mRNA concentration measured in experiment 1, and lower apo-B protein concentration and mRNA expression measured in experiment 3, indicate for a lower production of VLDL particles. Apo-B is essential for the transport of lipids packaged into VLDL and chylomicrones. MTP function is required for the assembly of Apo-B containing plasma lipoproteins. MTP transports lipid molecules from the endoplasmic reticulum membrane where they are synthesized, to developing lipoprotein particles in the endoplasmic reticulum lumen. The assembly of VLDL particles in the liver is believed to occur in two steps; first lipid is transferred by the MTP to apo-B during translation, and second, the apo-B containing precursor particles fuse with triacylglycerol droplets to form mature VLDL (Shelness and Sellers 2001). Hepatic levels of components such as cholesteryl ester and triacylglycerol also play a role in modulating VLDL formation (Mason 1998). Therefore, we suggest that the effect of soy protein on triglyceride concentration is mediated by a reduced endogen triglyceride synthesis and a diminished apo-B synthesis resulting in a reduced VLDL assembly and secretion.

In experiment 3, triglyceride concentration in the liver, plasma, VLDL, LDL and HDL was significantly lower in the rats fed soy protein isolate, pea protein or lupin protein compared to rats fed casein. These observations are in agreement with Iritani et al. (1985, 1986, 1996), who reported that the hepatic and plasma triglyceride content of the plant protein fed rats were significantly lower compared to animal protein fed rats. They also reported, that in the groups fed gluten and soybean protein compared with the groups fed casein or fish protein, the triglyceride contents were more markedly affected than the lipogenic enzyme activities. Another finding of our study was triacylglycerol-lowering effects of pea and lupin proteins like soy protein. Lupin protein lowered triacylglycerol concentration in plasma and VLDL more than soy protein but the triacylglycerol concentration in the liver were similar.

We suggest that like soy protein pea and lupin protein also lower triglyceride synthesis. But further experiments are required to find out the mechanism.

In both experiments, rats fed fish protein had significantly lower Triglyceride concentrations in plasma, VLDL, LDL and HDL compared to rats fed casein. These results are in agreement with Tsai and Huang (1999) who reported similar serum and VLDL triglyceride concentration in hamsters fed ethanol washed soy protein isolate or fish protein based diets. Recent studies with rats (Murata et al. 2004, Ait Yahita et al. 2005) also found a reduction in plasma and VLDL triacylglycerol concentrations in groups fed fish protein compared with groups fed casein and soybean protein, respectively. The hypotriglyceridemia primarily resulted from reduced amounts of circulating triglyceride carrying lipoproteins ($\delta <$ 1.006kg/L) and confirms other reports on the effect of fish protein in spontaneously hypertensive rats (Ait Yahita et al. 2004), rabbits (Bergeron et al. 1992), and premenopausal women (Gascon et al. 1996). Hypotriglyceridemia could possibly be caused by a diminished synthesis of TG in liver, an increased catabolism of fatty acids or a diminished secretion of triglycerides from liver via VLDL. The measure of SREBP-1c mRNA concentration along with the mRNA concentrations of the SREBP-1c target genes such as FAS, G6PDH and the Delta 6-desaturase and the activity of FAS and cDNA array analysis of acyl CoA carboxylase were not indicative of an inhibition of lipogenesis. But feeding fish protein probably enhanced fatty acid catabolism. As cDNA array results have shown lots of genes involved in fatty acid catabolism were up regulated by fish protein. Carnitine palmitoyl transferase (CPT) I and II, short chain acyl CoA dehydrogenase, medium chain acyl CoA dehydrogenase, and long chain acyl CoA dehydrogenase, which are involved in mitochondrial β oxidation of fatty acid were higher expressed in rats fed fish protein. There results are in agreement with Wergedahl et al. (2004) who reported higher activity of CPT-I in wistar rats fed fish protein. Acyl CoA esters can not cross the mitochondrial membrane, and their entry to the mitochondrion is the major point for control and regulation of the β-oxidation flux (Eaton 2002). Transfer across the mitochondrial membrane is achieved by transference of the acyl group from CoA to carnitine. This is accomplished by CPT-I on the outer membrane and by CPT-II on the inner face of the inner membrane. Twice expression of CPT-I in the liver of rats fed fish protein explains higher fatty acid catabolism in rats fed fish protein. Mitochondrion is the major site of fatty acid oxidation. In spite of mitochondria fatty acids are also oxidized in peroxisomes and microsomes. Higher expression of CYP4A1, CYP4A3, and CYP4A6 indicates higher rate of microsomal ω-hydroxylation. Higher expression of acyl CoA oxidase and 3-ketoacyl thiolase a + b indicates higher peroxisomal β-oxidation of fatty acid. Therefore, we suggest that

hypotriglyceridaemia observed in the fish protein-fed rats could at least be partially the result of increased oxidation of fatty acids. A former study with rabbits has found a higher lipoprotein lipase activity in the animals fed fish protein compared to animals fed soybean protein (Bergeron et al. 1992). Therefore hypotriglyceridaemia observed in the fish protein-fed rats could also be caused by a stimulation of plasma triglyceride clearance.

Another important finding is that most of the genes up-regulated by fish protein feeding like CPT-I, CPT-II, MCAD, Thiolase B, CYP4A1, CYP4A6, CYP7A1 are PPAR alpha target genes. Because PPAR alpha mRNA was not affected by feeding fish protein, the increased expression of PPAR alpha down stream genes was not due to a higher PPAR alpha expression. Hence, the up-regulation of PPAR alpha down stream genes should be the result of the activation of PPAR alpha protein by its agonist. PPARs mediate the effects of small lipophilic compounds such as long chain fatty acids and their derivatives on transcription of 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 mainly expressed in tissues exhibiting high rates of β-oxidation such as liver, heart, kidney and muscle (Desvergne and Wahli 1999). PPAR-α agonists increase the β-oxidation of fatty acids and therefore diminish the pool of fatty acids available for triglyceride synthesis and incorporation into VLDL. PPAR-α is involved in the regulation of peroxisomal β-oxidation (ACO, Thiolase B), mitochondrial ω-hydroxylation (CYP4A1, 4A6-Z), mitochondrial β-oxidation (CPT-I, CPT-II, MCAD), (Mandard et al. 2004, Dreyer et al. 1992).

In experiment 2, most remarkable differences by the animal proteins were observed in their effects on triglycerides. An interesting result of our study was the triglyceride-lowering effect of pork protein compared to casein. The extent of the reduction of the triglyceride concentration was 28% in plasma, 31% in the VLDL and 27% in LDL fraction and 46% in the liver. A previous experiment also found distinct differences between dietary proteins such as egg albumin, casein and wheat gluten on the concentration of triacylglycerols in liver of rats in which egg albumin has hypotriglyceridemic and wheat gluten hypertriglyceridemic action when compared to casein (Sugiyama et al. 1996). Although the authors did not explain these effects, their findings indicate a protein-mediated influence on the metabolism of triglycerides. Concentrations of triglycerides in liver and plasma are mainly dependent on the rate of hepatic lipogenesis (Foufelle et al. 1996). We therefore suggest that pork protein might have reduced the synthesis of triglycerides in the liver when compared to casein. Although we did not measure the mature fraction of SREBP-1c in nucleus or mRNA expression in the liver,

the reduced activities of FAS and G6PDH in the liver suggest diminished lipogenesis via SREBP-1c (Brandsch et al. 2006). This study therefore proposes that pork protein lowered fatty acid synthesis in the liver compared to casein via SREBP-1c mediated pathway.

### 4.4 Bile acid excretion

The liver centrally regulates whole body cholesterol excretion through the production and secretion of bile (Sautier et al. 1983). Several mechanisms may explain the hypocholesterolemic effect of soy proteins. Enhanced faecal steroid excretion which is the major route for cholesterol excretion may be one mechanism that explains the cholesterol lowering effect of soy protein (review by Potter 1996). In fact, a number of studies have shown that feeding soybean protein diets increases the faecal excretion of both neutral and acidic steroids in rats and rabbits (Potter 1996). Nagaoka et al. (1997) reported that lower serum cholesterol concentrations in rats as a result of soy feeding were associated with increased fecal excretion of total steroids. Wright and Salter (1998) reported an increase in bile acid excretion in hamsters fed intact soy protein compared to animals fed casein. They also reported a significant correlation between soy intake and bile acid excretion. In an earlier study, Nagata et al. (1982) also found increases in faecal steroid excretion in rats with dietary soy protein compared to casein. Sugano et al. 1982 reported an increase in faecal bile acid excretion and a reduction in hepatic cholesterol content with soy consumption compared to dietary casein.

Dietary soy protein enhanced bile acid excretion compared to the casein as we observed in our first two experiments, this may be partially responsible for the lower cholesterol concentration in the liver of rats fed soy protein. In first two experiments the expression of cholesterol 7 α hydroxylase was not different between the two groups. In our third experiment we did not measure the faecal bile acid excretion but the expression of cholesterol 7 α hydroxylase was significantly lower in rats fed soy protein compared to casein. This may be explained by the observation that in postprandial state both synthesis of cholesterol as well as bile acids were reduced by soy protein.

Fish protein also enhanced bile acid excretion compared to casein as we found in our second study. This higher excretion of bile acid was supported by higher expression of cholesterol 7 alpha hydroxylase, which is key enzyme involved in bile acid synthesis. In previous studies feeding fish protein, as compared with casein, was shown to increase faecal excretion of bile acids (Lapre et al. 1989, Iritani et al. 1985).

## 4.5 Amino Acids Concentrations in Plasma and Dietary Proteins

The difference in amino acid composition of dietary proteins can be reflected in the free amino acid pattern of plasma and some tissues either directly or indirectly. Based on this assumption, the relationship between the concentrations of plasma cholesterol and plasma free amino acids have been studied in humans (Sanchez et al. 1988), minipigs (Hagemeiser et al. 1990), rabbits (Kurowska and Carroll 1995), and rats (Horigome and Cho 1992). Barth et al. (1990) reported in pigs significant differences of postprandial plasma concentrations for 8 amino acids (cysteine, valine, methionine, leucine, tyrosine, lysine, tryptophan, and arginine) depending on whether they consumed a meal containing casein or isolated soy protein. They reported that the postprandial plasma amino acid pattern corresponds to the amino acid composition of the dietary proteins. We also observed that plasma amino acids concentrations of most of the amino acids were influenced by the type of dietary proteins and these observations were prominent in postprandial state (experiment 3). In experiment 3, plasma concentrations of cysteine, tyrosine, arginine, valine, leucine and lysine correspond to the amino acid composition of the dietary proteins.

Studies dealing with the effects of different dietary proteins from plant and animals sources on lipid metabolism suggest that specific amino acids could be responsible for the several effects observed (Kritchevsky et al. 1982, Sugiyama et al. 1986, Morita et al. 1997, Wergedahl et al. 2004). Sautier et al. (1983) reported that there was a significant correlation between the serum cholesterol concentration and the content of tyrosine, glutamic acid, cysteine, or alanine in dietary proteins in rats fed cholesterol-free diets containing one of the four types of proteins. They also reported that the serum cholesterol concentration could be significantly correlated with the content of proline, methionine, or alanine in dietary proteins when eight types of dietary proteins were used (Sautier et al. 1986). It has been suggested that the differences in plasma amino acid composition could affect cholesterol metabolism by altering hormone concentrations (Forsythe et al. 1986).

It has been suggested that amino acids such as methionine, glycine, arginine or lysine could be responsible for the different effects of dietary proteins on the lipid metabolism (Sugiyama et al. 1996, Sugiyama et al. 1997, Giroux et al. 1999, Gudbrandsen et al. 2005). Kurowska and Carroll (1995) reported that lysine and methionine contents of the diets seemed closely correlated with their hypocholesterolemic effect. Methionine was shown to elevate serum cholesterol concentration (Sugiyama et al. 1986). However, methionine

supplementation to a soy protein diet did not abolish the hypocholesterolemic effect of soy protein relative to casein (Kern et al. 2002, our third experiment, Shukla et al. 2006 b), suggesting that some factor other than methionine may be responsible at least in part for the cholesterol lowering effect of soy protein. Sugiyama et al. (1996) reported significantly positive correlation between the plasma cholesterol concentration and the plasma methionine or valine concentration. It was suggested that the higher ratio of methionine to glycine in casein may be responsible for the elevation in serum cholesterol (Morita et al. 1997), and glycine supplementation to a casein based diet lowered the serum cholesterol concentration in rats (Sugiyama et al. 1986).

In the present experiments the glycine and arginine contents of the soy protein diet were higher than that of the casein diet, and methionine and lysine content of the soy protein diet were lower that of the casein diet yielding lower methionine to glycine and lysine to arginine ratios in the soy protein diet. It was suggested that the increased serum cholesterol level that occurs with casein feeding was caused by the high ratio of lysine to arginine and methionine to glycine in casein (Morita et al. 1997, and Kritchevsky and Czarnecki 2000, Wergedahl et al. 2004). The dietary lysine to arginine ratios in the current study were 2.3 in the case of casein and 0.8 in the case of soy protein, and methionine to glycine ratios were 1.5 in the case of casein and 0.4 in the case of soy protein, favouring a cholesterol lowering effect by soy protein. Zhang and Beynen (1993) reported that the concentrations of cysteine and glycine in the diet were negatively associated with plasma and liver cholesterol concentrations. Similarily, significant negative correlations have been reported between serum cholesterol and dietary cysteine or alanine (Sautier et al. 1986, Sugiyama and Muramatsu 1990). In our study, cysteine and alanine concentration of soy protein were higher compared to casein. Thus, the amino acid composition of dietary proteins may be responsible in part for the effect of the protein source on liver cholesterol levels.

It was expected from previous reports that the higher proportion of methionine in casein would contribute to a higher plasma homocysteine, which is a risk factor for the development of atherosclerosis (Nehler et al. 1997, Toborek et al. 1995). Homocysteine is a sulphur-containing amino acid and is believed to act as an activator of SREBP-2 and thereby stimulates the gene expression of HMG-CoA reductase (Woo et al. 2005). Despite a significantly higher plasma concentration of methionine in rats fed casein compared to rats fed soy protein isolate, the homocysteine concentration in plasma did not differ between the two groups. Plasma homocysteine therefore provide no explanation for the observed activation of

SREBP-2. Ni et al. (1998) also observed no differences in serum homocysteine concentration in between soy protein isolate and casein fed apolipoprotein E-deficient mice.

However, it is more likely that soy protein peptides could be responsible for the observed effects. Alpha and alpha′ subunits from 7S soy globulin have been identified as peptides from soy protein that may regulate cholesterol homeostasis in HepG2 cells (Lovati et al. 2000). It seems not very probable that the remaining isoflavones in the ethanol-washed soy protein isolate may contribute to the observations made, because their concentration is extremely low compared to the concentrations normally used for induction of hypolipidemia (e.g. Mullen et al. 2004, Ali et al. 2004). However, it cannot be totally excluded that the remaining isoflavones may, at least in part contribute to the observations made.

When comparing the amino acids found in the dietary proteins extracted from meat with those of casein the greatest differences were observed for alanine, arginine, cysteine, glycine and aspartic acid (higher concentration in the meat proteins than in casein) and phenylalanine, glutamic acid proline, serine and tyrosine (lower concentration in the meat proteins than in casein). The ratios of methionine to glycine and lysine to arginine were also lower in proteins extracted from different meat compared to casein. The marked differences in the amino acid composition of the dietary proteins were not directly reflected in plasma. The most striking difference was observed for taurine, which was lowest in rats fed casein and highest in rats fed pork protein. Previous findings have shown that taurine supplementation reduced the concentration of triacylglycerols in liver and plasma of rats (Yan et al 1993, Park and Lee 1998). Since cysteine is a precursor of taurine, high intakes of cysteine might enhance taurine conjugation and, thus, have cholesterol lowering activity. Nanami et al. (1996) reported a hypocholesterolemic action of taurine supplemented diet in rats fed a high cholesterol diet. Another effect observed in rats fed diets containing proteins of beef, pork and turkey meat was reduction of the plasma homocysteine concentration compared with rats fed diets containing casein or soy protein. Plasma homocysteine concentrations are associated with the risk of cardiovascular and cerebrovascular disorders resulting from atherosclerosis (Clarke et al. 1991, Refsum et al. 1998, Welch et al. 1998). The reduction of plasma homocysteine concentrations by the animal proteins used in this study could therefore be of some physiologic relevance. The mechanisms underlying the homocysteine-lowering effects of these proteins are unknown, and require further investigation.

Fish protein when compared with casein had higher cysteine, aspartic acid, glycine, alanine, arginine content and lower serine, glutamic acid, proline, tyrosine, valine and phenyl

alanine contents. Fish protein had lower ratios of lysine to arginine and methionine to glycine. Fish protein lowered plasma homocysteine concentration. Plasma taurine concentration was significantly higher in rats fed fish protein compared to casein. Taurine is used for bile acid conjugation and may facilitate bile acids excretion in faeces. Moreover, taurine itself may enhance the biotransformation of cholesterol to bile acids, which may be partially responsible for the triacylglycerol lowering effect observed by fish protein feeding.

Pea and lupin proteins used in experiment 3, had higher cysteine, arginine and glycine, concentrations (about twice in the plant proteins than in casein). Valine, lysine and proline concentration of the plant proteins were lower compared to casein. The ratios of lysine to arginine and methionine to glycine were also lower in the plant proteins compared to casein. The plasma concentrations of arginine, asparagines and taurine were higher in the rats fed plant proteins and plasma concentrations of leucine, threonine, valine and tyrosine were lower compared to casein. Therefore, we suggest that dietary amino acids are at least partly involved in the cholesterol and triglyceride lowering effect observed by these proteins but we suggest that methionine might not be involved in the effects of these proteins on lipid metabolism observed in this study because we adjusted methionine concentrations of all plant proteins to a similar level as of casein in third study.

### 4.6 Final Thoughts:

When discussing the relevance of the effects observed in this rat study for human nutrition, several experimental points must be considered. Although rodents have been often used as an animal model to study the effects of dietary proteins on the metabolism of lipids (Terpstra et al 1982, Madani et al. 1998, Sugiyama et al. 1997, Ascencio et al. 2004, Gudbrandsen et al. 2005), it should be noted that they differ in their lipid metabolism in several regards from humans. For instance, they have generally lower concentrations of lipids (triglycerides, cholesterol) in plasma than humans and they carry most of their cholesterol in HDL which is also in contrast to humans (Jawie et al. 2004). In this study, we used a diet containing 100 g of lard per kg as a fat source rich in saturated fatty acids and supplemented additionally 5 g of cholesterol per kg of diet to mimic western diets of humans. Nevertheless, plasma cholesterol concentrations in the rats were relatively low. Other groups added cholic acid as a hyperlipidemic agent which increases absorption of dietary cholesterol and inhibits hepatic conversion of cholesterol into bile acids and thus results higher lipid concentration in plasma (Madani et al. 1998, Murphy et al. 2005). As dietary proteins can exert different

effects on the cholesterol metabolism in normolipidemic and in hyperlipidemic rats (Madani et al. 1998), it would be interesting to study the effects of dietary proteins in hyperlipidemic models in future studies.