THE EFFECTS OF FISH OIL ON LIPID AND LIPOPROTEIN METABOLISM IN F_1B AND GOLDEN SYRIAN HAMSTERS

MARION CORNISH

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The Effects of Fish Oil on Lipid and Lipoprotein Metabolism in F_1B and Golden Syrian Hamsters

By

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Abstract

We previously determined that fish oil induced hyperlipidemia in F_1B hamsters. In this study, we investigated the influence of fish oil enriched diets on lipid and lipoprotein metabolism in F_1B and GS hamsters to determine if the response was preserved within animal strain. Hamsters were fed either fish oil, a diet high in monounsaturated fatty acids, or a diet with an n-6:n-3 fatty acid ratio of 5. Fish oil induced hyperlipidemia to a much greater extent in F_1B compared to GS hamsters. Fish oil fed F_1B hamsters had milky plasma containing chylomicron-like particles which was not observed in GS hamsters. The concentration of plasma cholesterol, triglycerides, free cholesterol, and cholesterol esters were significantly higher in fish oil fed F_1B hamsters compared to GS hamsters. Feeding a diet with an n-6:n-3 ratio of 5 markedly decreased all lipid parameters in F_1B and GS hamsters compared to the fish oil diet. However, this diet increased LDL-cholesterol concentrations compared to the diet rich in monounsaturated fatty acids, suggesting that even a small amount of fish oil in the diet has a deleterious effect on the plasma lipoprotein profile. The presence of milky plasma, and elevated triglyceride concentrations suggested an inhibition of clearance of triglyceride-rich lipoproteins in F_1B hamsters. Thus, we investigated the activity of LPL, MTTP, LDL-receptor mRNA expression, and the protein expression of apoB and apoE to determine if diet or animal strain had an effect on these aspects of lipoprotein clearance. LPL activity, MTTP activity, and LDL-receptor mRNA expression were unaffected by diet, however LPL activity was lower in F_1B hamsters. In addition, the protein expression of apoB and apoE were altered by both diet and animal strain. In conclusion, comparison of F_1B and GS hamsters allowed us to attribute the fish oil induced hyperlipidemia to diversity in animal strain.

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List of Abbreviations

ACAT	Acyl Coenzyme A: Cholesterol Acyltransferase
ApoB	Apolipoprotein B
ApoCII	Apolipoprotein CII
ApoE	Apolipoprotein E
ALP	Atherogenic Lipoprotein Phenotype
CVD	Cardiovascular Disease
CE	Cholesterol Ester
CETP	Cholesterol Ester Transfer Protein
DHA	Docosahexaenoic Acid
EPA	Eicosapentaenoic Acid
FC	Free Cholesterol
HDL	High-Density Lipoprotein
LPL	Lipoprotein Lipase
LDL	Low-Density Lipoprotein
LDLr	Low-Density Lipoprotein Receptor
MTTP	Microsomal Triglyceride Transfer Protein
MUFA	Monounsaturated Fatty Acid
n-3	Omega-3
n-6	Omega-6
PMSF	Phenylmethanesulfonylfluoride
PUFA	Polyunsaturated Fatty Acid
SFA	Saturated Fatty Acid

SRE	Sterol Regulatory Element
TG	Triglyceride
VLDL	Very-Low-Density Lipoprotein

Chapter 1

Introduction

1.0 The Atherogenic Lipoprotein Phenotype

The atherogenic lipoprotein phenotype (ALP) is a collection of pro-atherogenic lipoprotein abnormalities which include moderately raised fasting triglyceride (TG) concentrations (1.5-4mmol/L), decreased high density lipoprotein (HDL)-cholesterol (<1.1mmol/L), and the predominance of small, dense low-density lipoprotein (LDL) particles, estimated to represent greater than 40% of total plasma LDL (Austin, 1988; Minihane *et al.*, 2000; Khan *et al.*, 2002). The observed hypertriglyceridemia is a direct result of defects in TG metabolism. Impaired clearance of TG-rich lipoproteins and more than adequate lipid substrates for the production of TG aids in an increase in the secretion of hepatic TG-rich very-low-density lipoprotein (VLDL) (Packard & Shepherd, 1997). In addition, elevated plasma TG levels results in a shunting of TG to LDL and HDL, lipoproteins that do not normally carry large amounts of TG.

The presence of TG-rich LDL is due to the facilitated catabolism of LDL by hepatic lipase. The resultant LDL particle is smaller and denser than the usual LDL particle. It has been reported by several investigators that the predominance of small, dense LDL particles is an independent risk factor for the development of cardiovascular disease (CVD), mainly due to an increase in oxidative susceptibility further contributing to foam cell formation (Austin, 2000). In combination, hypertriglyceridemia, small, dense LDL particles, and decreased HDL cholesterol concentrations characteristic of the ALP confer a 3-6 fold increase in CVD development (Griffin *et al.*, 1994). There is substantial evidence which has shown that the consumption of fat is directly linked to an ALP and the risk of CVD development.

1.1 Dietary Fats and Cardiovascular Disease

Dietary fats are known to be linked to the development of chronic diseases through the diet-induced regulation of lipid and lipoprotein metabolism. The ALP is a form of dyslipidemia commonly associated with obesity, Type II diabetes, insulin resistance and CVD (Khan *et al.*, 2002). More specifically, the quality of fat in the diet is as important as the quantity of fat consumed. The relationship between dietary fat and CVD is due mainly to its impact on the plasma lipid and lipoprotein profile. Saturated fats are detrimental to cardiovascular health as they increase LDL-cholesterol concentrations, promote the formation of atherosclerotic lesions, and the development of an ALP (Harris, 1989). Unsaturated fatty acids, on the other hand, namely monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids have been deemed cardioprotective due to their plasma lipid lowering capabilities and ability to improve an ALP (Harris, 1989). Thus, in recent years, a great deal of clinical and epidemiological research has been focused on the consequences of the enrichment of diets with MUFA and the essential PUFA, namely the omega-6 (n-6) and omega-3 (n-3) fatty acids.

1.1.2 Monounsaturated Fatty Acids and the Plasma Lipid Profile

Monounsaturated fatty acids are commonly found in the diet in the form of oleic acid (18:1). It was originally thought that supplementation of MUFA in the diet exerted neutral effects on the plasma lipid and lipoprotein profile, and thus were not as favourable a fatty acid for the reduction of hypercholesterolemia (Keys *et al.*, 1965; Hegsted *et al.*, 1965). More recent studies however, have shown that there are favourable effects of MUFA on the plasma lipid profile when they are substituted for dietary

saturated fatty acids (SFA) (Mattson & Grundy, 1985; Katan *et al.*, 1995). Much of the evidence for the beneficial effects of MUFA arises from studies of Mediterranean populations and the Mediterranean Diet. This population shows a low prevalence of CVD despite a dietary fat intake level of ~35%, obtained from MUFA mainly in the form of olive oil (Willett *et al.*, 1995). More recently, data from the Nurses Health Study also demonstrate that there are cardiovascular benefits attributed to high intakes of MUFA (Hu *et al.*, 1997).

The beneficial effects of MUFA arise from their capability to decrease both total and LDL-cholesterol without the development of hypertriglyceridemia at increasingly high concentrations (Mensink & Katan, 1992). It has been reported for example, that serum TG concentrations can decrease in normolipidemic or hypertriglyceridemic subjects on a high-MUFA diet (Mattson & Grundy, 1985; Grundy & Vega, 1988). This effect is reported to be independent of other dietary fatty acids, and occurs in a dose-dependent manner (Yu *et al.*, 1995; Gill *et al.*, 2003). In a study by Couture *et al.* (2003), LDL-cholesterol concentrations in men were decreased by 16% relative to baseline measurements in subjects on a MUFA diet. Several studies in adults and children have provided further support for these findings (Wahrburg *et al.*, 1992; Howard *et al.*, 1995; Kris-Etherton *et al.*, 1999; Hodson *et al.*, 2001; Nicklas *et al.*, 2002; Rajarani *et al.*, 2002). In addition, the cholesterol-lowering properties of MUFA have also been shown in children and adolescents with familial hypercholesterolemia (Guelesserian & Widhelm, 2001).

1.1.3 Polyunsaturated Fatty Acids and the Plasma Lipid Profile

The beneficial effects of PUFA are associated with both the n-6 and n-3 subclasses. The n-6 and n-3 PUFA also differ however, in their effects on the plasma lipid and lipoprotein profile. Some of the earliest studies examining the influence of dietary fat on plasma lipids have shown that n-6 PUFA, namely linoleic acid, lower serum cholesterol concentrations. This effect is of particular notice when n-6 fatty acids are substituted for dietary saturated fat (Kinsell *et al.*, 1953). These results are supported by several investigators who have found a decrease in the LDL-cholesterol fraction upon supplementation with n-6 fatty acids (Vega *et al.*, 1982; Mattson & Grundy, 1985; Mensink & Katan, 1989; Katan *et al.*, 1995). Omega-6 fatty acids have also been shown to reduce TG concentrations in hypertriglyceridemic patients (Grundy, 1975; Chart *et al.*, 1974). These results however, remain inconsistent. The effects of n-6 PUFA on the plasma lipid profile are primarily seen in a positive light, however it has been shown that high intakes of these fatty acids may reduce HDL-cholesterol concentrations, which can be a potential threat to cardiovascular health (Vega *et al.*, 1982; Shepherd *et al.*, 1978, Jackson *et al.*, 1984).

In contrast to the n-6 PUFA, n-3 PUFA have a distinct and consistent TG-lowering effect (Harris, 1989). Supplementation of n-3 fatty acids has been shown to decrease both VLDL assembly and apoB secretion (Harris, 1989; Wilkinson *et al.*, 1998; Kendrick & Higgins, 1999). Their effects on LDL-cholesterol concentrations however, remain inconsistent. In combination, and in appropriate proportions, n-6 and n-3 PUFA have the capability of significantly decreasing the risk of CVD development.

Consequently it is the ratio of n-6 and n-3 fatty acids that appears to be very important (Jump & Clarke, 1999).

1.1.4 The Omega-6 to Omega-3 Fatty Acid Ratio

Current diet trends in the Western world are more conducive to the development of chronic diseases such as CVD, Type II diabetes, and the associated hypertension and insulin resistance (Grimble, 1998). The Western population as a whole, particularly in North America does not benefit from the potentially anti-atherogenic properties of n-6 and n-3 fatty acids due to extremely high fat consumption. The current n-6 to n-3 ratio (n-6:n-3 ratio) peaks at 20-30:1 due mainly to an increase in the consumption of foods such as vegetable oil, which is increasingly rich in n-6 fatty acids (Simopoulus, 2002). This is a major concern as the n-6 and n-3 fatty acids compete for the elongase and desaturase enzymes required for breakdown and synthesis into eicosanoid products. Arachidonic acid is synthesized from n-6 fatty acids, and is the substrate for eicosanoids which play an important role in immune function, platelet aggregation, and cell proliferation. More specifically, n-6 derived eicosanoids, products of arachidonic acid, are potent inducers of platelet aggregation and inflammation (Simopoulos, 1999). It has been shown that enrichment of cells with arachidonic acid, particularly in red blood cells and platelets will increase the production of thromboxanes, prostaglandins and leukotrienes, and this can have deleterious effects on cardiovascular and respiratory health (Weber *et al.* 1989). In contrast to n-6 derived eicosanoids, those derived from n-3 fatty acids are considered to be anti-inflammatory and anti-aggregatory. Thus an excess of n-6 fatty acids in the diet

may overpower the metabolism of n-3 fatty acids and promote an inflammatory, as opposed to an anti-inflammatory responsive system.

There is international consensus that the current n-6:n-3 ratio of 20-30:1 needs to be lowered (Simopoulus, 2002). Current recommendations from the World Health Organization are to maintain an n-6:n-3 ratio of 5:1 to 10:1. The Canadian Government recommends 4:1 to 10:1. The optimal ratio remains controversial, with some recommendations as low as 2:1, or an equal amount of both n-6 and n-3 fatty acids (Simopoulos, 2002). The importance of decreasing the n-6:n-3 ratio for the improvement of cardiovascular health was demonstrated in the Lyon Heart Study (de Lorgeril *et al.*, 1994). This dietary intervention study provided its subjects with a linoleic to alpha-linolenic acid ratio of 4:1. At the end of a two-year period on this diet, they found an astounding 70% decrease in total mortality (de Lorgeril *et al.*, 1994). It has also been shown that lowering the n-6:n-3 ratio from 20:1 to 6:1 was associated with a decrease in the prevalence of Type 2 diabetes in Indian populations (Raheja *et al.*, 1993). Furthermore, it has been suggested that one of the mechanisms behind the positive influence of n-3 fatty acid supplementation is the reduction of the n-6:n-3 ratio and the subsequent decrease in arachidonic acid-derived eicosanoids within the body.

1.1.5 Fish Oil and the Plasma Lipid and Lipoprotein Profile

In focusing on decreasing the n-6:n-3 ratio, one of the main objectives is to increase the consumption of n-3 fatty acid containing products. One such product that has recently gained a lot of attention is fish oil. The advantageous effects of fish oil consumption are due to its relatively high concentrations of the marine derived n-3 fatty

acids eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid (Kagawa *et al.*, 1982; Dyerberg, 1986; Drevon, 1992). Bang *et al.* (1972) first recognized the importance of these fatty acids in 1972. These authors found that the decreased incidence of ischemic heart disease in Greenland Eskimos, despite relatively high dietary fat intake, was attributed to marine n-3 fatty acid intake.

Daily supplementation with fish oil has been shown to substantially decrease the development of CVD (Harris, 1997; Hu *et al.*, 2002). To date, the GISSI (GISSI Prevenzione Investigators, 1999) is the largest randomized controlled trial to examine the effects of the supplementation of 4g per day of EPA and DHA to patients who had previously experienced myocardial infarction. This study showed a significant 10-15% reduction in the cumulative rate of all-cause death and non-fatal myocardial infarction after a period of 3.5 years. There is further evidence that has shown an n-3 fatty acid dependent prevention of arrhythmias and sudden cardiac death (Jones, 2002; Lemaitre *et al.*, 2003).

The mechanisms by which fish oil improves cardiovascular function are numerous and include decreases in platelet aggregation (He, 2002), improvement in endothelial function (Mori & Beilin, 2001, Carroll & Roth, 2002), and the upregulation of lipid oxidation and simultaneous down-regulation of lipid synthesis (Carroll & Roth, 2002). Fish oil has also been shown to alter the plasma lipid and lipoprotein profile. Fish oil has been consistently shown to reduce TG concentrations in both normal and hypertriglyceridemic individuals (Phillipson *et al.*,, 1985; Harris, 1989; Sirtori *et al.*, 1997; Williams *et al.*, 1998). This is significant as elevated TG concentrations are considered to be an independent risk factor for the development of CVD (Braunwald *et*

al., 2001). Fish oil supplementation not only decreases fasting TG concentrations, but postprandial plasma TG concentrations are especially susceptible to chronic n-3 fatty acid consumption (Sanders *et al.*, 1997; Harris, 1988; Roche & Gibney, 1996). The reductions in plasma TG concentrations are mainly due to their influence on the synthesis and secretion of TG-rich lipoproteins. Decreased hepatic TG synthesis, TG secretion, and assembly of chylomicrons and VLDL are characteristic properties of fish oil (Nossen *et al.*, 1986; Nestel *et al.*, 1984; Wilkinson *et al.*, 1998).

Although fish oil has been shown to decrease plasma TG concentrations, the recommendation of fish oil for use as an anti-atherogenic therapy is still controversial. The effects of fish oil on total plasma and LDL-cholesterol concentrations remain varied. There are reports that normolipidemic patients ingesting fish oil have reductions in LDL-cholesterol concentrations. On the other hand, both normolipidemic and hyperlipidemic individuals ingesting fish oil have higher LDL-cholesterol concentrations (Illingworth *et al.*, 1984; Nester *et al*, 1984; Sullivan *et al*, 1986; Hsu *et al.*, 2000). Fish oil-induced elevations in plasma total- and LDL-cholesterol concentrations have been consistently reported in humans and are thought to be attributed to pre-existing dyslipidemia such as hypertriglyceridemia and Type II diabetes (Radack *et al.*, 1990; Farmer *et al.*, 2001). Since elevated total plasma- and LDL-cholesterol concentrations are independent risk factors for CVD, it is important to understand the regulation of cholesterol metabolism by fish oil.

1.2 Triglyceride-Rich Lipoprotein Metabolism and the Role of Dietary Fats

1.2.1 Hypertriglyceridemia and the Postprandial Response

Hypertriglyceridemia is indicative of abnormal postprandial lipemia and commonly accompanies the development of Type II diabetes and obesity. The presence of exaggerated plasma TG concentrations is also highly associated with CVD (Austin, 1991). It is only recently however that hypertriglyceridemia has been implicated as an independent risk factor for the development of CVD (Gotto *et al.*, 1998; Brewer *et al.*, 1999; Miller *et al.*, 2000). A meta-analysis conducted in 1996 using 17 population based studies revealed that increased plasma TG concentrations were associated with a significant risk for the development of CVD, initiating the adoption of elevated TG concentrations as an independent risk factor (Hokanson & Austin, 1996). Hypertriglyceridemia mainly arises from alterations in postprandial lipid and lipoprotein metabolism, which affects the synthesis, secretion, and clearance of TG-rich lipoproteins, namely chylomicrons and VLDL.

The postprandial state consists of the metabolic processes that occur after the ingestion, digestion, and absorption of a meal (Roche *et al.*, 1999). Most individuals will spend approximately 12 hours of their day in this state. Typically, plasma TG concentrations will increase shortly after a meal is ingested, and will not return to post-absorptive concentrations until several hours after the last meal of the day (Bergeron & Havel, 1995). Accumulating evidence indicates that dietary fat has a large influence on postprandial metabolism, which includes the aforementioned synthesis, secretion, and clearance of TG-rich lipoproteins.

Saturated fatty acids have been shown to prolong the accumulation of VLDL in the plasma during the postprandial period (Bergeron & Havel, 1995), significantly increasing the development of dyslipidemia. Monounsaturated fatty acids have proven to be a double-edged sword, with reports of little or no effect on postprandial metabolism compared to SFA (Roche *et al.*, 1992), or beneficial effects with the inhibition of hypertriglyceridemia upon supplementation (Mensink & Katan, 1992). PUFA have proven to be a positive influence on postprandial metabolism where individuals consuming diets rich in SFA had higher concentrations of chylomicrons and decreased clearance rates than those individuals consuming diets rich in n-6 or n-3 PUFA (Weintraub *et al.*, 1988; Demacker *et al.*, 1991). Longer term studies running for 15 and 16 weeks reveal that n-3 fatty acids are most effective at reducing postprandial TG concentrations (Agren *et al.*, 1996; Roche & Gibney, 1996).

While postprandial TG concentrations are important determinants for CVD risk, evidence also suggests that fasting TG-concentrations are also influenced by dietary fat intake and should be taken into consideration. Consumption of n-3 PUFA for example has been shown to exert a dose-dependent hypotriglyceridemic effect on fasting plasma TG concentrations (Sanders *et al.*, 1983; Blonk *et al.*, 1990; Schmidt *et al.*, 1990).

1.3 The Synthesis and Secretion of Triglyceride-Rich Lipoproteins

There are two main postprandial pathways by which TG-rich lipoproteins are metabolized. These include the exogenous pathway where chylomicrons are synthesized in enterocytes from dietary fat and cholesterol and act as a lipid shuttle between the gut and blood, with the liver as their final destination. The second is the endogenous

pathway, which involves the synthesis of hepatic VLDL from dietary TG returned to the liver by chylomicrons, and hepatically available TG. Both pathways involve lipoprotein formation from apoB and microsomal triglyceride transfer protein (MTTP), and can potentially be regulated by dietary fat.

1.3.1 Apolipoprotein B and the Synthesis and Secretion of TG-rich Lipoproteins

Apolipoprotein B is a key apoprotein involved in the synthesis of both chylomicrons and VLDL. There are two forms of apoB that exist in mammals. ApoB100 is the full length protein, and is synthesized exclusively by human liver, while apoB48 is a truncated version of apoB100 and is synthesized in the small intestine (Davidson, 2000). Two major steps are considered to be involved in the synthesis of these TG-rich lipoproteins, which include the synthesis of apoB and the presence of MTTP (Hussain, 2000; Cartwright & Higgins, 2001). The first step in TG-rich lipoprotein synthesis involves the translocation of apoB100 and apoB48 through the endoplasmic reticulum membrane in hepatocytes and enterocytes respectively (Berriot-Varoqueaux *et al.*, 2000). The addition of lipid begins co-translationally with the formation of a small apoB-containing lipoprotein particle. This process is crucial for the synthesis of TG-rich lipoproteins as apoB is an essential structural component of both VLDL and chylomicrons (Wang *et al.*, 2003).

There are several common mutations found in the human apoB gene that reveal its importance in lipoprotein metabolism. These are most often due to either an insertion or deletion at the signal peptide region (Vincent *et al.*, 2002). Hypobetalipoproteinemia is a result of a defect in the secretion of apoB, resulting in low plasma concentrations of

apoB-containing lipoproteins (Vincent *et al.*, 2002). Hypercholesterolemia is a result of defects in the binding and clearance of apoB-containing lipoproteins (Vincent *et al.*, 2002). Individuals carrying this mutation have highly elevated plasma LDL-cholesterol concentrations and are at an increased risk for the development of CVD (Vincent *et al*, 2002). An excess of apoB-containing particles has been shown to be a main trigger in the atherogenic process (Genest *et al*, 1992; Lamarche *et al*, 1997; Wallidus & Jungner, 2001).

1.3.1.1 Dietary Fats and Apolipoprotein B

The effects of SFA intake on apoB secretion parallel those seen in plasma lipid concentrations. Myristic, palmitic, and arachidic acids for example, are shown to significantly increase apoB secretion compared to controls, where arachidic acid has the most potent effect in HepG2 cells (Arrol *et al.*, 2000). These authors also find that MUFA (oleic and palmitoleic acid) can significantly increase apoB secretion above control values. On the other hand, several studies have shown that diets rich in MUFA significantly reduce apoB concentrations (Wahrburg *et al.*, 1991; Aro *et al.*, 1998; Gill *et al.*, 2003; Desroches *et al.*, 2004). Kinetic studies examining VLDL apoB in hypercholesterolemic adults revealed that apoB concentrations were significantly reduced by increasing the MUFA content of the diet (Gill *et al.*, 2003). Similar kinetic studies also report an effect of a high MUFA diet on VLDL apoB100 (Desroches *et al.*, 2004).

In comparison to MUFA, n-3 fatty acids, particularly those from fish oils, are known to decrease apoB secretion (Matsumoto *et al.*, 1987). The hypotriglyceridemic effects of n-3 fatty acids mentioned in the previous section are mainly attributed to the

inhibition of VLDL synthesis and secretion. Omega-3 fatty acids are thought to exert these properties through an increase in intracellular apoB degradation (Wong *et al.*, 1989). This has been supported by several studies in both HepG2, rat and hamster hepatocytes cell lines (Ribeiro *et al.*, 1991; Wang *et al.*, 1993; Brown *et al.*, 1997; Kendrick & Higgins, 1999; Fisher *et al.*, 2001). In hamster hepatocytes for example, Kendrick and Higgins (1999) demonstrate that the primary site of apoB degradation by dietary fish oils is the endoplasmic reticulum. Studies by Wang *et al.* (1993, 1994) also show that both EPA and DHA stimulate hepatic apoB degradation. This phenomenon also occurs in the intestine where apoB48 secretion is inhibited in hepatocyes isolated from rats fed EPA and DHA (Brown *et al.*, 1999).

In humans, the effects of n-3 fatty acids are not as consistent. In men with visceral obesity, fish oil supplementation was shown to significantly lower hepatic secretion of apoB by 29% compared to placebo (Chan *et al.*, 2003). Similar results have also been reported in hyperlipidemic patients given 1g/day of n-3 fatty acids for 10 weeks (Shidfar *et al.*, 2003). However, in hypertriglyceridemic patients with diabetes, apoB production was unresponsive to fish oil supplementation (Fisher *et al.*, 1998). Inconsistencies among studies are likely due to differences in study design which include the time and amount of supplementation and method of apoB measurement. Further work into this area is quite relevant as apoB has recently been identified as an independent predictor of the risk for heart disease (Lamarche *et al.*, 1996).

1.3.2 Microsomal Triglyceride Transfer Protein (MTTP) and the Synthesis and Secretion of TG-rich Lipoproteins

1.3.2.1 VLDL Synthesis and Secretion

MTTP is located both hepatically and intestinally and transfers lipid from the endoplasmic reticulum membrane to apoB. The lipidation of nascent apoB is a critical step in the formation of VLDL and is catalyzed by MTTP (Cartwright & Higgins, 2001). Additional TG is added in this step of VLDL assembly by fusion of apoB with preformed lipid droplets (Berriot-Varoqueaux *et al.*, 2000). The exact mechanisms by which this occurs are not full understood, but several investigators have shown a physical interaction between apoB and MTTP (Hussain *et al.*, 1997; Hussain *et al.*, 1998; Wu *et al.*, 1996; van Greevenbroek *et al.*, 1998). This binding is an essential component of VLDL assembly. Compounds that disrupt the binding of MTTP to apoB can result in the decrease of apoB secretion by 75-80% (Hussain *et al.*, 1998; Bakillah *et al.*, 2000). The involvement of MTTP in VLDL synthesis indicates that it plays a critical role in regulating plasma lipoprotein metabolism. The importance of MTTP is observed in patients with abetalipoproteinemia, where mutations in MTTP result in defective lipid transfer activity and absence of apoB-containing lipoproteins in the plasma (Davidson & Shelness, 2000; Gordon & Jamil, 2000).

1.3.2.2 Dietary Fats and VLDL Synthesis and Secretion

It has been clearly shown that dietary fatty acid composition has an influence on VLDL synthesis, secretion, and MTTP activity (Salter *et al.*, 1998). Diets rich in SFA

increase VLDL synthesis and result in prolonged accumulation of postprandial VLDL (Bergeron *et al.*, 1995). Monounsaturated fatty acids reportedly have a similar effect as SFA on TG synthesis, despite their beneficial effects on plasma lipoprotein concentrations. Several investigators have found that oleic acid can induce a higher rate of TG synthesis, and can activate MTTP activity more so than linoleic or palmitic acids (Field *et al.*, 1988; van Greevenbroek *et al.*, 1998). Similarly, in hamsters, Bennett *et al.* (1995) have shown that increasing the amount of triolein in the diet produces a linear increase in hepatic MTTP mRNA concentrations.

Fish oils are known to exert opposite effects on VLDL synthesis in comparison to SFA and MUFA. They have been shown to suppress endogenous VLDL secretion (Ikeda *et al.*, 2001; Harris, 1997; Bordin *et al.*, 1998; Williams *et al.*, 2004). Kinetic studies in humans have indicated that n-3 PUFA inhibit VLDL synthesis and secretion (Harris, 1990). Similarly, feeding studies show that n-3 fatty acids reduce hepatic secretion of TG, cholesterol, and apoB in VLDL (Ribiero *et al.*, 1991; Brown *et al.*, 1999). In animals, n-3 PUFA have been shown to decrease TG synthesis through the inhibition of lipogenic enzymes such as 1,2-diacylglyceroltransferase (Rustan *et al.*, 1988, 1989). In rats fed diets with either 10% w/w fish oil or olive oil, those rats on the olive oil diet showed a higher rate of TG and VLDL formation than those on the fish oil diet (Lottenberg *et al.*, 1992). Similarly, in hypertriglyceridemic men, Westphal *et al.* (2000) found that treatment with n-3 fatty acids reduced fasting VLDL-TG concentrations by 44% compared to other dietary fats. Fish oils have also been shown to decrease apoB100 secretion from HepG2 cells (Wong *et al.*, 1989).

While several investigators have demonstrated that dietary fat can alter MTTP activity, the effects of specific fatty acids have not been well studied. It has been shown in hamsters however, that the response of hepatic MTTP mRNA to dietary fat is dose-dependent (Bennett *et al.*, 1995). This group found that triolein, or a mixture of triolein and linoleic acid reduced hepatic MTTP mRNA levels compared to a mixture of triolein and SFA. The capability of fish oils to lower VLDL synthesis and secretion suggests that these n-3 PUFAs may also influence hepatic MTTP activity. However, fish oils are thought to primarily act on reducing the synthesis of apoB. Their role in regulating MTTP activity has not yet been examined.

1.3.3.3 Chylomicron Synthesis and Secretion

Dietary fat is absorbed in the intestine by enterocytes, and ultimately assembled into chylomicrons (Cooper *et al.*, 1997). The translocation of apoB through the endoplasmic reticulum in enterocytes and subsequent lipidation of this nascent particle by MTTP is as important and occurs in the same manner as VLDL synthesis (Hussain, 2000). Since MTTP is located both hepatically and intestinally, it is obvious that MTTP is involved in the assembly of chylomicrons and plays an important role in fat absorption from the gut (Salter *et al.*, 1998).

1.3.3.4 Dietary Fats and Chylomicron Synthesis and Secretion

The metabolism of chylomicrons is also influenced by dietary fat, although this phenomenon has not been as thoroughly studied as the effects of dietary fat on hepatic lipoproteins. In general, SFA are known to increase chylomicron synthesis, while PUFA

reduce postprandial chylomicron concentrations (Harris, 1997). One of the first observations regarding the influence of dietary fat on chylomicron metabolism was seen by Tso *et al.* in 1984. This group found that palmitic acid stimulated the output of chylomicron-sized lipoproteins while linoleic acid led simply to the formation of fewer, larger chylomicron-sized particles. Field *et al.* (1988) also found that oleic acid treatment of CaCo-2 cells reduced the rate of TG synthesis. This work was further supported by van Greevenboek *et al.* (1996) who found that oleic acid had a much greater impact on the suppression of *de novo* TG synthesis than linoleic and palmitic acids. Increasing the amount of fat in the diet also influences chylomicron metabolism. For example, as the quantity of fat in the diet increases, the ability of rabbit enterocytes to produce and secrete apoB48, TG, and cholesterol esters (CE) also increases (Cartwright & Higgins, 1999). It is interesting that the rate of chylomicron synthesis was least influenced by dietary fish oil. Omega-3 fatty acids found in fish oil have also been shown to lower TG synthesis and secretion from CaCo-2 cells (Murthy *et al.*, 1990). However, it can be argued that chylomicron and VLDL assembly are not similarly affected by fish oil, as there is no direct evidence to indicate an inhibitory effect of fish oil on chylomicron synthesis. It has been shown that fish oil does not slow TG absorption or chylomicron secretion in rats (Harris, 1997) or rabbit enterocytes (Cartwright & Higgins, 1999).

It is thought that the influence of fatty acids on chylomicron metabolism may reflect differences in the activity of MTTP towards TG with specific fatty acid compositions. The effect of specific fatty acids on intestinal MTTP activity however, has not been thoroughly investigated.

1.4 Triglyceride-Rich Lipoprotein Clearance

1.4.1 The Role of Lipoprotein Lipase

Lipoprotein lipase (LPL) is one of the most critical enzymes involved in lipoprotein metabolism (Braun & Severson, 1992; Enerback & Gimble, 1993; Mead *et al.*, 2002). LPL is located on the luminal surface of the endothelium, attached by highly charged heparin-sulfate proteoglycans. The function of LPL is to catalyze the hydrolysis of the TG-rich lipoproteins, chylomicrons and VLDL. LPL generates the release of free fatty acids in the form of monoacylglycerol and non-esterified fatty acids (Potts *et al.*, 1991) for uptake by adipose tissue for storage, or by the muscle to be oxidized and used for the production of energy (Pentikainen *et al.*, 2002).

Interestingly, LPL is considered to be both a pro-atherogenic, and an anti-atherogenic enzyme. More specifically, a macrophage that expresses LPL is pro-atherogenic, whereas muscle and adipose tissue LPL is anti-atherogenic. The pro-atherogenicity of LPL was originally proposed by Zilversmit in 1973. This hypothesis is based on the fact that LPL produces chylomicron remnants and LDL, the remnant products of chylomicrons and VLDL respectively. Both of these have been implicated in the development of atherosclerosis (Botham *et al.*, 1997 Phillips *et al.*, 1993; Karpe & Hultin, 1995). It has been shown for example, that chylomicron remnants may be taken up as efficiently as LDL by blood vessel walls and significantly contribute to atherosclerotic lesions (Shaikh *et al.*, 1991; Mamo & Wheeler, 1994; Proctor & Mamo, 1998; Grieve *et al.*, 1998).

The majority of the LPL enzyme is located on muscle and adipose tissue, the major sites of synthesis of the enzyme, and plays a protective role in atherosclerosis, mainly through the clearance of circulating lipoproteins (Mead *et al.*, 2002). The protective effect of LPL has been demonstrated by several investigators (Mead *et al.*, 2002). Hydrolysis of lipoproteins by LPL drives the lipoprotein profile in a non-atherogenic direction by increasing HDL-cholesterol concentrations (Mead *et al.*, 1999, 2002). Several clinical studies have also shown that there is a strong correlation between the development of CVD and a decrease in LPL activity (Kastalein *et al.*, 2000; Hitsumoto *et al.*, 2000). For example, Hitsumoto *et al.* (2000) demonstrated that patients with coronary atherosclerosis had significantly lower pre-heparin serum LPL mass than healthy patients. Furthermore, as previously mentioned, the delayed clearance of chylomicron remnants from the blood, which is associated with a decrease in LPL activity, is thought to be associated with the possible development of atherosclerotic lesions (Mahley *et al.*, 1991; Groot *et al.*, 1991; Patsch *et al.*, 1992). The anti-atherogenic properties of LPL are particularly evident in patients with Familial Chylomicronemia Syndrome (Merkel *et al.*, 2002). Here, individuals who are homozygous for LPL-deficiency are found to develop early atherosclerosis (Benlian *et al.*, 1996), with dramatic increases in TG concentrations above 11.3mmol/L, chylomicronemia, and decreases in HDL-cholesterol. Diminished LPL activity in adipose tissue has also been implicated in the development of hypertriglyceridemia in obese individuals and those who have type II diabetes (Mead *et al.*, 1999; Mamputer *et al.*, 1997). Additional evidence for the anti-atherogenic potential of LPL is apparent in studies that examine the overexpression of LPL (Shimada *et al.*, 1996; Yagyu *et al.*, 1999). These investigators have shown

normalization of an atherogenic lipoprotein phenotype when LPL is overexpressed in LDL receptor and apolipoprotein E (apoE) knock-out mice.

Lipoprotein lipase is regulated by a vast number of hormones such as insulin, glucocorticoids, and adrenaline (Braun & Severson, 1992; Enerback & Gimble, 1993). Adipose tissue LPL for example, is exclusively activated by the action of insulin during the fed state (Eckel *et al.*, 1995; Panarotto *et al.*, 2002; Arasaradnan *et al.*, 2002). In order for maximal activation of LPL however, apolipoprotein CII (apoCII) is necessary (Mead *et al.*, 2002). This apoprotein is located on the surface of both chylomicrons and VLDL. Without the presence of apoCII, LPL exhibits a much lower activity both *in vitro* and *in vivo* (Cryer *et al.*, 1981; Wang *et al.*, 1992).

1.4.1.1 Dietary Fats and Lipoprotein Lipase

LPL has also been shown to be regulated by dietary fat, however the effects of specific dietary fats on LPL activity are limited and much less established than the role of hormones. Upon comparison of diets rich in SFA or MUFA, Roche *et al.* (1998) found that LPL was not significantly different between these two diets. In addition, when used as a placebo, olive oil treated subjects had lower LPL activity than those fed fish oil (Khan *et al.*, 2002).

The majority of studies have examined the effects of n-3 fatty acids and fish oil on LPL activity and shown highly inconsistent results (Park & Harris, 2003). Moreover, the regulation of pre- and post-heparin LPL by dietary fats also appears to be partially dependent on whether an individual is in the fed or fasted state. Studies using various animal models such as rats, chickens, and pigs have not shown any effect of fish oil on *in*

vitro post-heparin LPL (Harris, 1987; Daggy *et al.*, 1987; Huff *et al.*, 1993). Several *in vitro* human studies have also reported that n-3 fatty acids do not enhance post-heparin LPL activity (Harris, 1988; Weintraub *et al.*, 1988; Desager *et al.*, 1989; Nozaki *et al.*, 1991; Lovegrove *et al.*, 1997). A recent study in 2003 by Park and Harris for example, examined the specific effects of EPA and DHA on LPL activity using a trace amount of radiolabelled lipid emulsion that tracks native chylomicrons. These investigators found that while EPA and DHA significantly increased pre-heparin LPL activity during the fed state, that both pre- and post-heparin LPL activity were unaffected in the fasted state. On the other hand however, fish oil has the capability of increasing LPL activity and thus contributing to the anti-atherogenic properties of fish oil (Kasim-Karis *et al.*, 1995; Harris, 1997; Khan *et al.*, 2002). Harris (1997) demonstrated in rats that chylomicron lipids were cleared at a much faster rate when the animals were fed fish oil compared to control animals who were not fed fish oil. Similarly, there have been reports of increases in LPL activity in both adipose tissue (Anil *et al.*, 1992; Benhizia *et al.*, 1994; Yoshiba *et al.*, 1999) and muscle (Herzberg & Rogerson, 1989) in rats fed diets rich in n-3 PUFA in comparison to those fed n-6 PUFA.

The explanation for the varied response of LPL activity is currently unknown. The activity of LPL as measured in the above mentioned studies however, is not necessarily a direct indicator of *in vivo* hydrolysis of chylomicrons and VLDL. The composition of chylomicrons and VLDL may also affect the rate of LPL mediated fatty acid release. Fish oil feeding to animals and humans for example results in chylomicrons that show decreased lipolysis by LPL (Botham *et al.*, 1997; Levy & Herzberg, 1999). It has also been shown that VLDL and chylomicrons compete for LPL activity where

chylomicrons are preferentially hydrolysed by LPL (Potts *et al.*, 1991; Karpe *et al.*, 1995). In individuals with elevated VLDL concentrations, the clearance of chylomicrons is partially inhibited (Grundy *et al.*, 1976; Goldberg & Merckel, 2001). Furthermore the LPL enzyme becomes saturated at VLDL concentrations above 5mmol/L, which will concurrently decrease both VLDL and chylomicron metabolism (Brunzwell *et al.*, 1973). Thus the lipid profile of an individual may exert a large influence on LPL activity. It has also been postulated that specific dietary fatty acids may modify the regulation of LPL by insulin (Sadur *et al.*, 1984). It is obvious then, that while LPL is key in regulating both the extent and duration of postprandial lipemia, its regulation by dietary fat is an area of interest and requires further investigation.

1.4.2 The Role of the LDL-Receptor

The LDL receptor (LDLr) is another key component in maintaining cholesterol homeostasis through the endocytosis of chylomicron remnants and LDL by the liver (Larsson *et al.*, 2004). The clearance of cholesterol-rich chylomicron remnants and LDL particles is accomplished by receptor-mediated endocytosis, fusion of LDL with lysosomes, and recycling of the LDLr to the hepatic cell surface. The ligand binding domain of the LDLr recognizes both apoB100 and apoE (Brown *et al.*, 1983; Russell *et al.*, 1989). ApoE-containing lipoproteins however have higher affinity for the LDLr than apoB-contaning lipoproteins (Innerarity *et al.*, 1978). This is thought to occur due to the multiple copies of apoE which can bind to several LDL receptors.

The importance of the LDLr and its role in the clearance of cholesterol-rich lipoproteins is apparent in individuals with the dominantly inherited disorder familial

hypercholesterolemia (FH). Here, dysfunctions in the LDLr lead to an accumulation of cholesterol-rich LDL in the plasma (7.8-13mmol/L in heterozygotes, and 16-31mmol/L in homozygotes) (Takahashi *et al.*, 2004). These individuals also develop premature atherosclerosis (Schuster *et al.*, 2002). Furthermore, the widely-used LDLr-knockout mice display severe hypercholesterolemia and the onset of premature CVD.

1.4.2.1 Dietary Fats and the LDL-Receptor

The amount, expression, and activity of the LDLr is primarily regulated by cholesterol, as established by Brown and Goldstein. Cholesterol regulates the expression of the LDLr via the binding of sterol regulatory element binding proteins to sterol regulatory elements (SRE) (Sudhof *et al.*, 1987). It has been shown that high intakes of cholesterol will simultaneously down-regulate the biosynthesis of the LDLr and increase the circulating pool of LDL (Beynen *et al.*, 1983; Russell *et al.*, 1983). On the other hand, when there is a shortage of hepatic cholesterol, cells will respond by upregulating the transcription of the LDLr (Ma *et al.*, 1986). Earlier studies propose that *in vivo* regulation of the LDLr is regulated by cholesterol flux only (Takagi *et al.*, 1989). More recent studies however, have also demonstrated the regulation of LDLr activity and mRNA abundance by dietary fatty acids (Kurushima *et al.*, 1995; Mistad *et al.*, 1996).
The saturated fatty acids, lauric (12:0), myristic (14:0), and palmitic (16:0) have been shown by several investigators to decrease LDLr activity, protein, and mRNA abundance (Woollett *et al.*, 1992, 1994; Kurushima *et al.*, 1995; Mustad *et al.*, 1996; Nicolosi *et al.*, 1997). The literature investigating the effects of unsaturated dietary fatty acids however, are much more controversial.

In cell culture, the presence of MUFA have been shown to significantly increase activity of the LDLr (Daumerie *et al.*, 1992; Rumsey *et al.*, 1995; Gill *et al.*, 2003). Studies in hamsters have also shown similar results (Kurushima *et al.*, 1995). In contrast to these findings however, Berry *et al.* (1992) demonstrated that LDLr status was unchanged in the presence of MUFA in fresh monocytes. The majority of studies have shown that supplementation of n-3 fatty acids, particularly fish oils, will decrease hepatic LDLr activity and mRNA abundance. Supplementation of fish oil in HepG2 cells and rat hepatocytes has been shown to down regulate the LDLr and inhibit LDLr-mediated uptake (Roach *et al.*, 1987; Wong & Paul, 1987). Similarly, Lindsay *et al.* (1992) measured LDLr activity in HepG2 cells both before and after fish oil supplementation. These authors found a 20% decrease in LDLr mRNA abundance and a 60% decrease in LDLr activity after fish oil supplementation. A more recent study in HepG2 cells by Pal *et al.* (2002) also found that LDLr binding potential, activity, protein, and mRNA levels concurrently decreased as the degree of unsaturation increased, where EPA had the greatest depressive effect.

This reported down-regulation of the LDLr by fish oil has also been investigated in several animal studies (Hayes *et al.*, 1988; Spady *et al.*, 1995). Fish oil feeding to miniature pigs has been shown to decrease LDLr activity (Huff *et al.*, 1993), while a 70% decrease in the amount of LDLr was seen in rabbits fed fish oil compared to sunflower oil (Wilkinson *et al.*, 1998). In contrast to these findings, there are that diets enriched with fish oil can enhance LDLr activity (Ventura *et al.*, 1989). Ventura *et al.* (1989) for example, found that rat chow enriched with 20% fish oil significantly increased LDLr activity.

The mechanism by which dietary fatty acids regulate the LDLr is as equally controversial as their direct effects on LDLr mRNA abundance and LDLr activity. It has been suggested that fatty acids regulated LDLr in a similar fashion as cholesterol, as PUFA have been shown to mediate SREBP expression (Worgall *et al.*, 1998). These authors found that increasing unsaturation led to an increase in the suppression of SRE-mediated gene expression of the LDLr. Others suggest that dietary fats exert their effects independently of cholesterol and do not influence SREBP expression (Field *et al.*, 2003). Another school of thought is that dietary fats regulate LDLr activity by affecting cholesterol ester and free cholesterol regulatory pools by influencing acyl-coenzyme-A acyl transferase (ACAT) activity, involved in the esterification of hepatic cholesterol (Spady *et al.*, 1993; Dietschy *et al.*, 1993; Yu-Poth *et al.*, 2004). Further investigation into the role of dietary fat in the regulation of the LDLr will give insight into the therapeutic potential of the upregulation of the LDLr and consequent decrease in plasma cholesterol concentrations.

1.4.3 The Role of Apolipoprotein E

Apolipoprotein E is indisputably the most important ligand for the clearance of TG-rich lipoproteins. ApoE is synthesized and secreted primarily by hepatocytes (Kolovou *et al.*, 2003), and is abundant in humans at levels of 5-8g/L (Hasty *et al.*, 1999). The main function of apoE is to serve as a ligand for hepatic cellular lipoprotein receptors, namely the LDLr , and subsequent uptake by the liver (Curtiss & Boisvert, 2000). ApoE is the main determinant of chylomicron and VLDL concentrations in the general population (Ordovas & Mooser, 2002) and accounts for approximately 30-50% of

TG-rich lipoprotein clearance (Kril *et al.*, 1983; Tomiyasu *et al.*, 2001). ApoE also facilitates the interaction of TG-rich lipoproteins with LPL (Asset *et al.*, 2001).

ApoE is considered to be an anti-atherogenic apolipoprotein and plays a large role in plasma cholesterol homeostasis. More specifically, apoE promotes the reduction of plasma cholesterol concentrations (Shimano *et al.*, 1992; Nikoulin & Curtiss, 1998). This is most apparent in patients with a deficiency in apoE, also known as Type III hyperlipoproteinemia (Mahley *et al.*, 1988). These individuals experience an accumulation of plasma chylomicrons and VLDL, both of which are risk factors for the development of CVD (Mahley *et al.*, 1988). The importance of apoE has also been revealed in apoE knockout mice. These mice have extremely high levels of plasma cholesterol compared with wild type mice and can lose their resistance to cholesterol feeding (Plump *et al.*, 1992; Zhang *et al.*, 1992). These mice have plasma cholesterol concentrations greater than 52mmol/L and develop complex atherosclerotic lesions (Piekrahita *et al.*, 1992). Interestingly, the re-introduction of apoE in experimental animals by such methods as bone marrow transplantation (van Eck *et al.*, 1997) or transgenics (Shimano *et al.*, 1992) has been shown to decrease plasma cholesterol concentrations. Transgenic mice overexpressing human apoE for example are known to be resistant to cholesterol feeding (Shimano *et al.*, 1992).

1.4.3.1 Dietary Fats and Apolipoprotein E

The regulation of apoE concentrations by the diet is a relatively new area of investigation. While diet is considered to be the main environmental challenger of apoE concentrations, the effects of specific dietary fats, especially fish oil, are not well studied

(Ribalta *et al.*, 2003). A recent study in humans by Buckley *et al.* (2004) has shown a 15% increase from a basal level of 30g/L in apoE concentrations in a fish oil fed group. In contrast however, it has been shown that meals rich in SFA show a non-significant tendency for high postprandial apoE concentrations (Mero *et al.*, 1998). Similarly, a recent study conducted in adult men found that ingestion of a meal enriched with SFA markedly increased the apoE content of TG-rich lipoproteins compared to MUFA and PUFA enriched meals (Jackson *et al.*, 2005).

One explanation for the inconsistent findings with respect to the regulation of apoE by dietary fat is the existence of apoE isoforms which are known to influence the plasma lipid and lipoprotein profile. ApoE isoforms result from three alleles: ε-2, ε-3, and ε-4, and determine not only the concentration and amount of apoE in the plasma (Larson *et al.*, 2000), but the affinity of lipoproteins for the LDLr (Ribalta *et al.*, 2003). It has been shown that the presence of apoE isoforms may account for up to 40% of the variation that is seen in circulating TG concentrations (Salah *et al.*, 1997). ApoE isoforms have a large influence on lipoprotein concentrations where individuals carrying the ε-2 isoform have lower plasma- and LDL-cholesterol, and higher HDL-cholesterol concentrations then ε-3 carriers, but ε-4 carriers have higher total and LDL-cholesterol and lower HDL-cholesterol than ε-3 carriers (Ribalta *et al.*, 2003). It has also been shown that ε-2 and ε-4 carriers have higher plasma TG concentrations compared to ε-3 carriers (Dallongeville *et al.*, 1992). Variations in apoE isoforms are also associated with an increased risk of premature atherosclerosis (Srinivasan *et al.*, 1999; Rubin *et al.*, 2002).

The effect of dietary fats on apoE in isoform carriers has been more commonly studied than in individuals with a normal apoE genotype (absence of apoE isoforms).

Some studies have shown for example that SFA intake worsens the lipoprotein profile (Campos *et al.*, 2001) and increases the postprandial response in ε-2 carriers (Weintraub *et al.*, 1987; Orth *et al.*, 1996), but not in ε-4 carriers. Others have shown that there is no difference in the plasma lipid response to dietary interventions across apoE genotype (Friedlander *et al.*, 2000). While apoE is key in regulating lipoprotein levels in the plasma and uptake by peripheral tissues, there is a great deal of uncertainty concerning the regulation of apoE by dietary fats.

1.5 The Hamster as an Animal Model for the Study of Lipid and Lipoprotein Metabolism

The hamster is considered to be an ideal animal model for the examination of lipoprotein metabolism due to its similarities to human lipoprotein metabolism (Nistor *et al.*, 1987; Spady *et al.*, 1998). Hamsters carry a significant proportion of their serum cholesterol in the LDL fraction, as do humans (Ohtani *et al.*, 1996). Hamsters also have plasma cholesterol ester transfer protein (CETP) activity as seen in humans (Ahn *et al.*, 1994), whereas rats and mice have virtually no plasma CETP activity (Ha & Barter, 1982). Most importantly, the secretion of TG-rich lipoproteins in hamsters is very similar to humans. Hamsters secrete apoB48 exclusively from the intestine, and apoB100 exclusively from the liver, whereas rats secrete both forms of apoB from the liver. It is also known that the level of hepatic cholesterol synthesis in hamsters is equivalent to humans (Spady & Dietschy, 1988; Woollett *et al.*, 1989), and that cholesterol metabolism is also partially regulated by the LDLr (Chen *et al.*, 1996; Remillard *et al.*, 2001). Hamsters also secrete bile that is relatively rich in cholesterol (Anderson *et al.*, 1986).

In addition to similarities in the blood lipid and lipoprotein profiles, hamsters also respond to dietary fat and cholesterol in the same manner as humans (Sullivan *et al.*, 1993). For example, serum LDL cholesterol concentrations increase in response to cholesterol and saturated fat (Woollett *et al.*, 1992). It has been shown however, that not all hamster strains respond similarly to dietary fat (Dorfman *et al.*, 2001). Different breeding laboratories such as Biobreeders and Charles Rivers labs produce two different hamster strains (Trautwein *et al.*, 1993). The GS hamster from Charles Rivers is an outbred strain, whereas the F_1B hamster from BioBreeders is an inbred strain. Compared to GS hamsters, F_1B hamsters have lower HDL concentrations, and a higher VLDL and LDL to HDL ratio (Trautwein *et al.*, 1993). F_1B hamsters also develop atherosclerotic lesions at much lower concentrations of dietary cholesterol (Kowala *et al.*, 1991). Hypercholesterolemia is much more easily induced in F_1B than GS hamsters (Nicolosi *et al.*, 1998). It is thought that the varied response to dietary fatty acids seen among hamster studies may reflect strain-specific differences. In any case, the hamster is a widespread and useful model for the examination of lipid and lipoprotein metabolism.

1.6 Genetics, Dietary Fats and CVD

It has long been established that genetics and family history play a significant part in the development of CVD. It is evident from the literature mentioned in the previous sections on polymorphisms in MTTP, LPL, apoB, apoE, the LDLr, and the variability in animal models, that heterogeneity in genetic background, and gene-nutrient interactions have a large influence on plasma lipid concentrations.

Some of the first studies to recognize the diversity in the plasma lipid profile were conducted by Ahren *et al.* (1957) who found marked heterogeneity among subjects in their plasma cholesterol response to dietary cholesterol intake. Furthermore, there are several theories which involve the existence of hypo- and hyper-responders to dietary fat intake (Jacobs *et al.*, 1981; Katan *et al.*, 1986). Phenotypic diversity in several animal models has also been shown to result in varied response to dietary fat intake (Bhattacharyya *et al.*, 1987; Overturf *et al.*, 1990). In humans, a study by Gylling *et al.* (1992) found that low baseline plasma LDL-cholesterol concentrations, apoE2 phenotype, and reduced bile acid synthesis were correlated with a low response to dietary cholesterol intake. While the most extensively investigated loci involved in gene-nutrient interactions studies are apoE, apoA4, apoB, LPL, and CETP (Ordovas *et al.*, 2002), there is an increasing amount of evidence that variation in intestinal cholesterol absorption, or hepatic cholesterol synthesis between individuals is responsible for the difference in response to dietary fat (Stein *et al.*, 2002). Further investigation into gene-nutrient interaction and heterogeneity in genetic background will give great insight into potential dietary therapies that may be moulded to an individual rather than a population, and could greatly decrease the variability in response to dietary fat.

1.7 Justification for this study

Fish oil has typically been shown to be beneficial for the plasma lipid profile by reducing TG concentrations, which are known to be an independent risk factor for the development of CVD. The effects of fish oil on plasma cholesterol concentrations however, remain controversial. Previous work in our lab investigated the effects of fish

oil on the regulation of plasma lipoproteins, particularly its effects on LDL-cholesterol concentrations, using the F_1B hamster as an animal model (de Silva *et al.* 2004). In order to determine the mechanisms by which fish oil was altering cholesterol metabolism, these hamsters were fed a low fat (5% w/w) or a high fat (20% w/w) fish oil diet for a period of two weeks. Surprisingly, fish oil did not reduce TG concentrations, but induced severe hyperlipidemia in these hamsters and had a deleterious effect on the plasma lipid and lipoprotein profile. F_1B hamsters fed the fish oil diet had highly elevated plasma-, VLDL- and LDL- cholesterol and TG concentrations and the presence of milky plasma. Thus the high level of n-3 fatty acids present in the fish oil diet were not effective in lowering plasma lipid levels. Fish oil-fed F_1B hamsters also had a decrease in LDLr mRNA expression, and a significant decrease in CETP activity.

The highly elevated plasma-, VLDL-, and LDL-lipid concentrations, the presence of milky plasma, and the alterations in cholesterol metabolism suggest that fish oil is regulating either the synthesis and secretion, or the clearance of lipoproteins in F_1B hamsters. Thus in this study, we investigated the mechanisms by which fish oil regulates lipoprotein metabolism in the unique F_1B hamster. We compared the effect of three specific diets: a fish oil diet, a diet rich in monounsaturated fatty acids (MUFA), and a diet with an n-6:n-3 ratio of 5 (N6:N3), using F_1B and GS hamsters. These three diets are considered to be cardioprotective and allow us to determine if the fish oil-induced hyperlipidemia is specific to fish oil, or if other fatty acids have this same effect. In addition, the fish oil-induced increase in LDL-cholesterol concentrations, compared to that seen in humans, presents us with a unique animal model to investigate the mechanisms by which fish oil is regulating cholesterol metabolism.

We also compared the F_1B hamster to the GS hamsters to determine if the response to fish oil was strain-specific. It is not known whether these two hamster strains respond similarly to dietary fish oil and this comparison is important to recognize whether genetic background plays an important role in diet-induced regulation of lipid and lipoprotein metabolism. It is well known that the development of CVD can be partially attributed to genetics, and we are aware that heterogeneity within genetic background in humans contributes to the varied response to dietary fatty acid supplementation. Therefore the location of polymorphisms between hamster strains would provide insight into diet-induced regulation of lipoprotein metabolism in humans.

The objectives of this study then were to determine how fish oil regulates lipid and lipoprotein metabolism in F_1B and GS hamsters, and if this metabolism is differentially regulated between animal strains. We investigated the role that fish oil plays in lipoprotein secretion by examining the activity of MTTP, and the protein expression of apoB. We also examined fish oils effects on lipoprotein clearance by examining LPL, ApoE, and the LDLr.

We found that fish oil feeding to F_1B and GS hamsters induced hyperlipidemia compared to these hamsters on the MUFA and N6:N3 diets. In addition, this dyslipidemia was much more prominent in F_1B than GS hamsters. We also find significant effects of diet and strain on several components involved in lipoprotein metabolism which include LPL activity, and the expression of apoB and apoE. The findings of our study highlight the importance of several factors in the regulation of lipoprotein metabolism such as n-6:n-3 balance in the diet, the effects of dietary fat on the synthesis, secretion and

clearance of TG-rich lipoproteins including LPL activity, apoB and apoE, and the role that genetic background may play in nutritional studies.

Chapter 2

Methodology

2.1 Animals and Diets

Male Bio F_1B and Golden Syrian hamsters (7 weeks old) were obtained from BioBreeders Inc. (Maryland, USA) and Charles Rivers Labs (Kingston, NY, USA) respectively. The animals were kept on a chow diet for one week prior to feeding special diets. After this equilibration period, animals in each strain were divided into one of three groups (n = 6, F_1B; n = 6, GS) and were fed one of three specified diets for a period of four weeks. These specified diets consisted of fat-free semi-purified diet (ICN Biomedicals, Ohio, USA) supplemented with 200g/kg (20% w/w) of either fish oil (FO) (Menhaden oil, Sigma – Aldrich, Ontario, Canada), high MUFA safflower oil obtained from a local supermarket (MUFA), or a diet designed to give the animals an n-6:n-3 fatty acid ratio of ~5 (N6:N3) using fish oil, safflower oil and lard. Due to the presence of 2.5g/kg cholesterol in the fish oil diet (as per Sigma Aldrich Menhaden Oil composition), cholesterol was added to the MUFA and N6:N3 diets in appropriate proportions. The diets were kept at –20°C and given daily *ad libitum*. The composition of the diets is given in Table 1. Food intake was measured daily and animal body weight was measured weekly. The animals were housed in individual cages in a single room. The room was lit from 0700 hrs to 1900 hrs with the temperature maintained at 21°C and humidity at 35 ± 5%. All procedures were approved by Memorial University's Institutional Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care.

After the four week feeding period, the animals were fasted for 14 hours prior to sacrifice. The hamsters were then anaesthetized with halothane. The livers were freeze-clamped in liquid nitrogen and stored at –70°C until further use. The intestines were also removed and snap-frozen in liquid nitrogen, and stored at –70°C until further use.

Table 1

Diet composition for the fish oil (FO), monounsaturated fatty acid rich (MUFA) and N6:N3 diets.*

	Diet		
g/kg	FO	MUFA	N6:N3
Casein	200	200	200
DL-Methionine	3	3	3
Sucrose	305	305	305
Maize Starch	190	190	190
Vitamin mix[a]	11	11	11
Mineral mix[a]	40	40	40
Fibre[§]	50	50	50
Fat	200	200	200
Cholesterol	2.5	2.5	2.5

* Semi-purified diet designed for a 200g/kg fat level

[a]Supplied in quantities adequate to meet nutritional requirements (National Research Council, 1995)

[§] Supplied as Alphacel non-nutritive bulk (ICN Biomedicals, Aurora, OH, USA)

2.2 Gas-Liquid Chromatography

Gas-liquid chromatography (GLC) analysis was conducted to determine the fatty acid composition of the fish oil, MUFA, and N6:N3 diets (Table 2). Lipids were extracted from the diets using chloroform-methanol (2:1 ratio) as described previously (Yokode *et al.* 1990). Lipids were then trans-methylated by adding 2mL of 6% sulphuric acid in methanol and heating at 65°C for three hours. Fatty acid-methyl esters were extracted from each sample by adding 1mL of deionized water and 1 mL of hexane. The top hexane layer was transferred to a clean, acid-washed tube and dried under nitrogen. The fatty acid methyl esters were dissolved in 10μL of carbon disulfide. Approximately 2μL of sample was placed into a glass vial insert and placed in a crimp vial (VWR, Ontario, Canada). Samples were run for 40 minutes on an Omegawax x320 (30m*0.32mm) column from Supelco (Sigma-Aldrich, Ontario, Canada) using a flame ionization detector (FID). The GLC parameters were set as follows: oven, 196°C; injector, 240°C; detector, 250°C. Fatty acids standards were used for quantification purposes.

Table 2

*Fatty acid composition for the fish oil (FO), monounsaturated fatty acid rich (MUFA), and N6:N3 diets (% of total fatty acids)**

Fatty Acid	Diet		
	FO	**MUFA**	**N6:N3**
14:0	15	0.8	4
16:0	17	15	23
16:1 n-7	10.3	1.5	4.5
18:0	7.0	9.1	7.8
18:1	6.2	53	41
18:2 n-6	1	18	12
18:3 n-3	0.5	0.8	-
20:4 n-6	6	1.6	4.5
20:5 n-3	12	0.2	1
22:5 n-3	7	-	0.3
22:6 n-3	18	-	1.9
SFA	39	24.9	34.8
MUFA	16.5	54.5	45.5
PUFA (n-6)	7	19.6	16.5
PUFA (n-3)	37.5	1	3.2

* Lipids were extracted from the diets and fatty acid composition was determined by GLC

2.3 FPLC Separation of Plasma

Plasma samples were separated on a Superose 6 Chromatography column (HR 10/30) (Amersham Biosciences, Quebec, Canada). Plasma samples obtained from F_1B and GS hamsters on the fish oil, MUFA, and N6:N3 diets were diluted to 200μL with HPLC water and filtered into inserts using a 0.22 micron syringe filter (Amersham Biosciences, Quebec, Canada) before injecting into the HPLC. The HPLC program consisted of a run time of 60 minutes and an injection volume of 75μL using a solvent system consisting of a 0.9% sodium chloride solution with 1% sodium azide. The plasma lipoprotein cholesterol profile was determined by using a post-column online enzymatic assay reagent (Cholesterol Liquicolor Kit, Stanbio, Texas, USA). 16mL of reagent was diluted with 0.02% sodium azide in 100mL of HPLC water and the flow rate for the cholesterol reagent was set at 0.1mL/minute (Cheema & Agellon, 1999).

2.4 Plasma Separation and Lipid Analysis

Fasting blood samples were collected by cardiac puncture into tubes containing EDTA. The plasma was then separated by centrifugation at 3000rpm for ten minutes and stored on ice at 4°C until further analysis. Plasma was allowed to sit overnight to allow chylomicrons to separate. Chylomicrons were then removed before lipoprotein separation. Individual lipoprotein fractions were isolated by sequential density gradient ultracentrifugation using a TL100 fixed angle rotor. The lipoproteins were separated according to specific densities of <1.006, 1.006-1.060, and >1.060 g/mL for VLDL, LDL (LDL + IDL), and HDL respectively (de Silva *et al.*, 2004). This was done using solutions which contained increasing amounts of NaCl for VLDL and LDL separation, or

NaBr for HDL separation, to adjust the density of the plasma. To isolate VLDL, plasma (0.5mL) was added to a NaCl solution (0.5mL) with a density of 1.0063 g/mL and centrifuged at 100,000 rpm at 16°C for 2.5 hours with an acceleration of 5 and a deceleration of 7. VLDL was then carefully removed and stored on ice at 4°C. To isolate LDL, a further 0.5mL of NaCl solution with a density of 1.12 g/mL was added to the remaining sample and mixed gently. The conditions for centrifugation for isolation of LDL remained the same as those for VLDL. After centrifugation, LDL was then carefully removed and stored on ice at 4°C. In order to isolate HDL, 0.5mL of NaBr solution with a density of 1.361 g/mL was added to the remaining sample and mixed gently. The sample was then centrifuged for 3.5 hours at 100,000 rpm at 16°C with an acceleration of 5 and a deceleration of 7. These lipoprotein fractions were stored on ice at 4°C and all analyses were performed within one week of plasma collection.

The plasma and isolated lipoproteins were analyzed for total cholesterol (Cholesterol Liquicolor Kit, Stanbio, Texas, USA), TG (mono- and diacylglycerol included) (Triglyceride Enzymatic Kit, Stanbio Labs, Texas, USA), and free cholesterol (FC) (Free cholesterol Enzymatic Kit, Wako Chemicals, Virginia, USA). Cholesterol ester concentrations were determined by subtracting free cholesterol concentrations from total cholesterol concentrations.

2.5 Hepatic Lipid Profile

Liver lipids were extracted using chloroform-methanol (2:1 ratio) as previously described and resuspended in 100μL of isopropanol (Folch *et al.* 1957). The liver lipids were analyzed for total cholesterol (Cholesterol Liquicolor Kit, Stanbio, Texas, USA),

TG (Triglyceride Enzymatic Kit, Stanbio Labs, Texas, USA), and FC (Free Cholesterol Enzymatic Kit, Wako Chemicals, Virginia, USA). Cholesterol ester concentrations were determined by subtracting free cholesterol concentrations from total cholesterol concentrations. Standards were also suspended in isopropanol for consistency.

2.6 Lipoprotein Lipase Assay

The LPL assay involved several steps including the release of LPL, preparation of the apoCII source, and the LPL enzyme assay. This assay measures the amount of free fatty acids released into the plasma by LPL. The LPL assay was performed using previously published methods (Nilsson *et al.* 1976; McAteer *et al.* 2003). F_1B and GS hamsters on the specified diets were anaesthetized using Somnatol. Lipoprotein lipase was released into the circulation by the direct injection of 250μL of heparin (250U/kg) to the heart. Heparin was allowed to circulate throughout the body for 5 minutes to release LPL into the blood stream. The addition of heparin was sufficient to act as an anti-coagulant, thus blood was then collected by cardiac puncture and centrifuged at 3000rpm for 15 minutes at 4°C to separate the plasma. The plasma was stored at –70°C for the LPL assay.

For the apoCII source, blood was obtained from a control hamster and incubated at 37°C for one hour. The serum was obtained by centrifugation of the blood at 1100 rpm for 20 minutes. The LPL enzyme was inactivated by heating at 56°C for one hour. Serum was stored at –20°C as the source of apoCII.

2.6.1 Preparation of the Stock Glycerol Emulsion

Tri [9,10-^{3}H] oleoyl glycerol (Amersham Biosciences, Quebec, Canada) was purified on a silica gel Whatman TLC plate using a hexane: diethyl ether: acetic acid (80:20:1) solvent system. Triolein was used as a standard, and bands were visualized using an iodine chamber. Tri [9,10-^{3}H] oleoyl glycerol was extracted from the silica gel and added to 2mL of choloroform:methanol (2:1) in a clean glass vial and vortexed for 2 minutes. An additional 1mL of the chloroform:methanol mixture was added to rinse the vial and the contents were transferred to a new vial.

Triolein (414mg) and phosphatidylcholine (40mg) were added to the vial containing the purified glycerol tri [9,10 3H] oleate and dried under nitrogen. Glycerol (30mL) was then added, and the mixture was sonicated (45 amplitude) for four minutes at one-minute intervals. The glycerol stock solution was stored at room temperature.

2.6.2 Lipoprotein Lipase Assay

For the LPL assay, an emulsion containing a 0.62M Tris-HCl solution with 5% BSA, pH 8.6, serum as a source of apoCII, and the glycerol stock emulsion in a ratio of 1:0.5:1 was prepared. Plasma (25μL) and 50μL of either 3.55M NaCl (hepatic lipase) or 0.15M NaCl (total lipase) were added to glass tubes and pre-incubated at 30°C in a shaking water bath for 30 minutes. In order to start the reaction, 60μL of the emulsion was added to each sample and incubated at 30°C in a shaking water bath for 60 minutes. The reaction was stopped by adding 1.625mL of a methanol:chloroform:heptane solution (1.45:1.23:1) and 0.525mL of 0.1M potassium carbonate-potassium borate buffer, pH 10.5. Samples were vortexed for 30 seconds and centrifuged at 1100rpm for 10 minutes

at room temperature. After centrifugation, 0.5mL of the upper phase was added to a scintillation vial with 18mL of scintillant. Radioactivity was counted in a scintillation counter.

2.7 Microsomal Triglyceride Transfer Protein Activity

MTTP activity was measured in liver and intestine samples from F_1B and GS hamsters on the fish oil, MUFA, and N6:N3 diets using a kit method (Roar Biomedicals, New York, USA). 100mg of liver or intestinal samples were homogenized in 1mL of homogenization buffer (1M Tris, pH 7.4, 5M NaCl, 0.5M EDTA, 100mM phenylmethanesulfonylfluoride (PMSF)) using a Teflon homogenizer at 4°C. The homogenate was then sonicated for 5 seconds at 1 minute intervals for 3 repetitions to completely disrupt the cells. The homogenate was then centrifuged at 9g for 5 minutes at 4°C, and the supernatant removed, aliquoted, and stored at –70°C. Protein concentration was determined using the Lowry protein assay (Lowry, 1951). Protein (150μg), 5μL of self-quenched donor, and 5μL of acceptor (reagents in the kit) were diluted to a final volume of 260μL with MTTP buffer (de Silva, 2004 b). The samples were incubated at 37°C for 2 hours in a shaking incubator. The fluorescence was read using a Spectramax Gemini Fluroescence spectrophotometer at an excitation wavelength of 465nm, and an emission wavelength of 535nm. This assay measures the MTTP-mediated transfer of lipid in a sample, the intestine and liver in this case. This transfer is determined by the increase in fluorescence intensity as the fluorescent lipid is removed from the self-quenched donor (mentioned above) to the acceptor. MTTP activity is subsequently expressed as pmol of lipid transferred, per hour, per milligram of tissue (pmol/hr/mg).

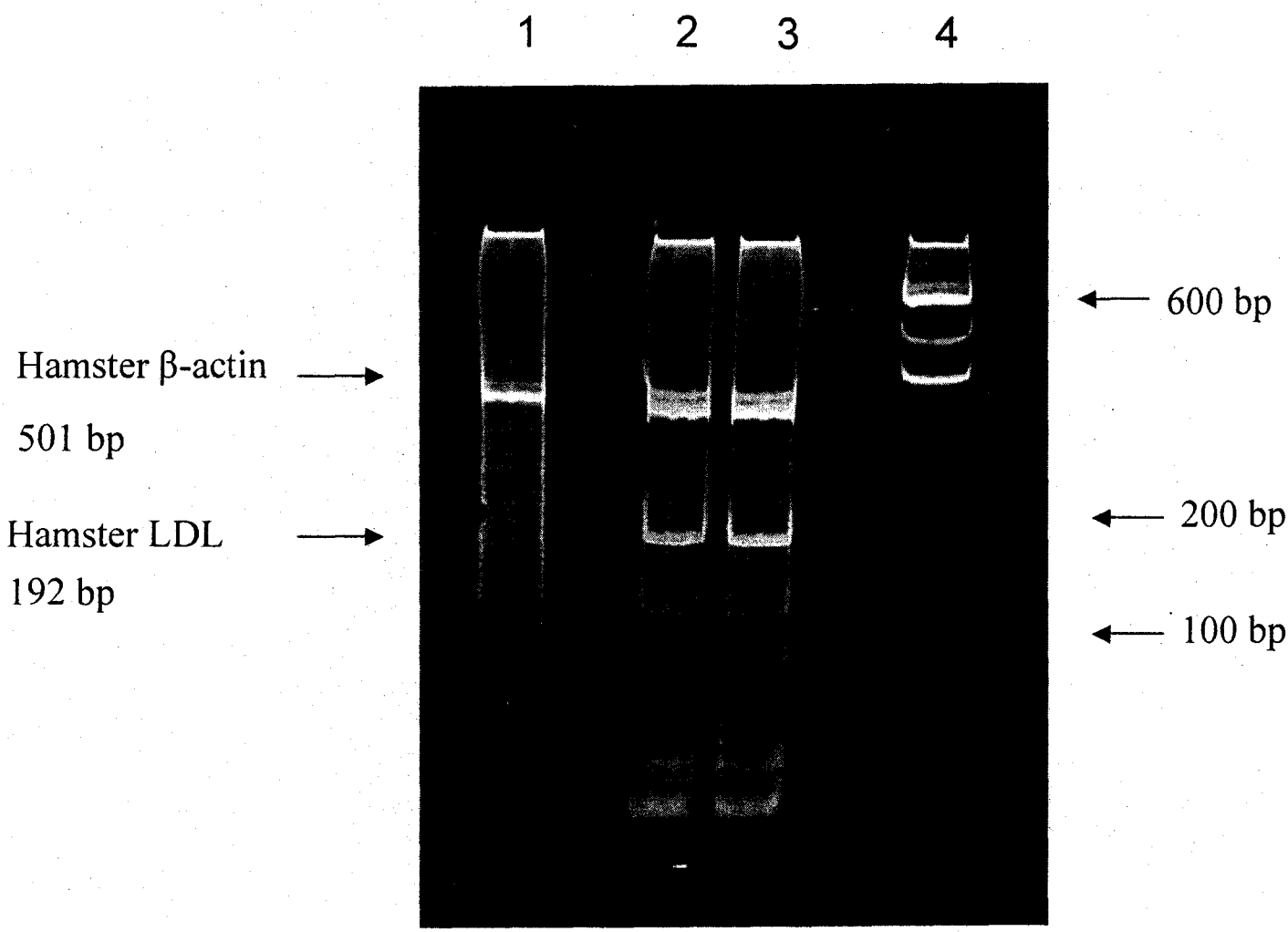
2.8 LDL receptor mRNA expression

RT-PCR was used to determine LDLr mRNA abundance in liver samples from F_1B and GS hamsters fed the fish oil, MUFA, and N6:N3 diets as per previously published methods in our lab (de Silva *et al.*, 2004). Total RNA from hamster livers was isolated using the FastPrep RNA kit Green, and the FastPrep FP120 instrument (Qbiogene, Carlsbad, CA). RNA samples were checked for integrity on a 1.5 % agarose gel using borate buffer.

Complementary DNA was synthesized by reverse transcription from 2μg of total RNA using Superscript II reverse transcriptase and used as templates for *in vitro* DNA amplification. Both the LDL-receptor and β-actin mRNA were amplified simultaneously in a multiplex system. The specific primers were as follows: hamster LDLr sense 5'-TCGGTGACAA-TGTCGCACCAAG-3', antisense 5'-GCTTCTGGTACACTGGTTGTC-3', hamster β -actin sense 5'-CATCGTACTCCTGCTTGCTG-3', antisense 5'-GCTACAGCTTCAC-CACCACA-3'.

PCR was conducted in the Genius PCR machine from Roche. The following conditions were used: initial heating to 94°C, steady at 94°C for 1minute, 2 minutes at 55°C, and 72°C for 3 minutes. The total number of cycles was chosen to remain within the exponential phase of the reaction at 30 cycles. PCR products were stored at 4°C and resolved on a 10% acrylamide gel in TBE buffer (Figure 2.1). The bands were quantified using the ChemiImager 4400 gel documentation system. LDL receptor mRNA expression was normalized against β -actin mRNA expression and expressed as relative units.

Figure 2.1: RT-PCR of the LDL-receptor and β-actin. Total RNA was reverse transcribed and cDNA was amplified by PCR for LDL-receptor and β-actin in a multiplex system as described in the materials and methods section. Lane 1 represents the amplification in the absence of reverse transcriptase, lane 2 and 3 represents the amplification of hamster β-actin and LDL-receptor in the presence of reverse transcriptase, and lane 4 shows the 100bp ladder.


1
2
3
4
Hamster β-actin
501 bp
Hamster LDL
192 bp
600 bp
200 bp
100 bp

2.9 Western Blot Analysis

The Western blot analysis was conducted by the use of previously published methods to measure the protein expression of apoB and apoE in plasma samples from F_1B and GS hamsters on the fish oil, MUFA, and N6:N3 diets (Burnette, 1981).

2.9.1 TCA Precipitate

TCA precipitation was performed to delipidate the plasma and to determine the amount of protein in each plasma sample. Plasma (5μL), deionized water (20μL) and 50% TCA (25μL) were added to an eppendorf tube. Samples were allowed to precipitate on ice for 10 minutes and were then centrifuged at 10 000g for 10 minutes. 250μL of 10% TCA was the added to each sample, and samples were again centrifuged at 10 000g for 5 minutes. The pellet was then resuspended in 10μL of 1M NaOH. Deionized water (80μL) was added to each sample to bring the final volume to 100μL. The samples were assayed for protein concentrations using the Lowry Method.

2.8.2 Western Blot Analysis for ApoB

Plasma samples containing 60μg protein from F_1B and GS hamsters fed various diets were run on a 6% SDS polyacrylamide gel (Calbiochem, California, USA). 25μL of a biotinylated protein ladder was used as a marker. The gel was run for 1 hour and 15 minutes at 200V. The proteins and marker were transferred to a nitrocellulose membrane (Trans-blot transfer medium, pure nitrocellulose membrane, 0.2uM, Biorad, California, USA) for 1.5 hours at 100V at 4°C. The membrane was then blocked for 1.5 hours in TBS buffer containing 5% (w/v) non-fat dried Carnation milk (local supermarket) and

probed overnight with goat polyclonal antibody against human apoB100 and apoB48 (Goat polyclonal antibody against human apoB, cat.no. 178467, Calbiochem, California, USA). The primary antibody dilution was 1:1000 in TBS buffer containing 0.05% Tween 20 (TBST). The membrane was rinsed the next day with TBST and bovine anti-goat IgG conjugated with horseradish peroxidase (1:5000 dilution with TBST) was added as a secondary antibody (Santa Cruz, California, USA). During the secondary antibody incubation, 2.5μL of HRP-conjugated Anti-biotin antibody (Calbiochem, California, USA) was added after one half hour to detect the biotinylated protein marker. After washing with TBST, reactions were detected using enhanced chemiluminescence reagent system (Santa Cruz, California, USA).

2.8.3 Western Blot Analysis for ApoE

Plasma samples containing 60μg protein from F1B and GS hamsters fed various diets were run on a 10% SDS polyacrylamide gel (Calbiochem, California, USA). 25μL of a biotinylated protein ladder was used as a marker. The gel was run for one hour and seven minutes at 200V. The proteins and marker were transferred to a nitrocellulose membrane (Trans-blot transfer medium, pure nitrocellulose membrane, 0.2uM, Biorad, California, USA) for 1.5 hours at 100V at 4°C. The membrane was then blocked for 1.5 hours in TBS buffer containing 5% (w/v) non-fat dried Carnation milk (local supermarket) and probed overnight with rabbit polyclonal antibody against human apoE (Dako, Denmark) The primary antibody dilution was 1:2000 in TBST. The membrane was rinsed the next day with TBST. Anti-rabbit HRP-linked antibody was added (1:5000 dilution) as a secondary antibody (Calbiochem, California, USA). During the secondary

antibody incubation, 2.5μL of HRP-conjugated anti-biotin antibody was added after one half hour incubation to detect the biotinylated protein marker (Calbiochem, California, USA). After washing with TBST, reactions were detected using an enhanced chemiluminescence reagent system (Santa Cruz, California, USA).

2.9 Statistical Analysis

The effect of diet type and animal strain were determined using 2-way ANOVA. A Newman-Keuls post-hoc test was used to test significant differences revealed by ANOVA. Values are group means (n = 6, F_1B; n = 6, GS) unless otherwise stated, and standard error of the mean (SEM). Differences were considered statistically significant when $P \leq 0.05$.

Chapter 3

Results

3.1 Food Intake and Body Weight

There was a significant effect of strain on body weight. GS hamsters were significantly heavier than F_1B hamsters initially, and this continued throughout the entire course of the study (Table 3). There was no effect of diet on body weight. There was no significant effect of diet or strain on food intake (Table 4).

3.2 Plasma Lipid Profile

Figure 3.1 depicts plasma samples collected after a 14-hour fast in F_1B hamsters on the fish oil and MUFA diet. F_1B hamsters on the fish oil diet only had milky plasma containing chylomicron-like particles. Milky plasma was not observed in GS hamsters on the fish oil, MUFA, or N6:N3 diet. Figures 3.2-3.5 show the plasma cholesterol, TG, FC, and CE concentrations from F_1B and GS hamsters on all three specified diets. There is a marked effect of both diet and strain on all four lipid parameters. Two-way ANOVA analysis revealed a significant effect of both diet ($P < 0.0001$) and strain ($P < 0.0001$) on plasma cholesterol, TG, FC, and CE. Due to the presence of a significant D x S interaction ($P < 0.0001$), one-way ANOVA was also performed.

F_1B hamsters on the fish oil diet had significantly higher plasma total cholesterol concentrations ($P < 0.001$) compared to F_1B hamsters on the MUFA and N6:N3 diets (Figure 3.2). Similarly, fish oil fed GS hamsters had significantly higher plasma total cholesterol concentrations ($P < 0.001$) than GS hamsters on the MUFA and N6:N3 diets. While fish oil feeding significantly increased total plasma cholesterol concentrations in both hamster strains, fish oil fed F_1B hamsters had significantly higher concentrations ($P < 0.001$) than fish oil fed GS hamsters. Plasma cholesterol concentrations in F_1B

Table 3

Initial and 4 week weight (g) for F_1B and GS hamsters on the fish oil (FO), monounsaturated fatty acid rich (MUFA) and N6:N3 diets

Animal	Diet	Initial BodyWeight (g)	Statistical Analysis		Body Weight after 4 weeks (g)	Statistical Analysis	
			Interaction	P-value		Interaction	P-value
F_1B	FO	99.3 ± 2.9	S	P < 0.0001	111 ± 3.4	S	P < 0.0001
	MUFA	103 ± 2.6			118 ± 2.5		
	N6:N3	98.1 ± 2.5			109 ± 3.7		
GS	FO	121 ± 1.9	S	P < 0.0001	125 ± 2.5	S	P < 0.0001
	MUFA	118 ± 1.7			126 ± 2.4		
	N6:N3	122 ± 2.1			130 ± 2.7		

Values are means SEM, (n=6 for F1B, n=6 for GS)
Groups were compared by two-way ANOVA, $p \leq 0.05$
D, S represent diet and strain respectively

Table 4

Average food intake for F_1B and Golden Syrian hamsters fed the fish oil (FO), MUFA or N6:N3 diets

Animal	Diet	Food Intake (g/day)	Statistical Analysis	
			Interaction	P-value
F_1B	FO	5.30 ± 0.25		
	MUFA	5.46 ± 0.30	S	P = 0.06
	N6:N3	5.04 ± 0.34		
			D	P = 0.63
GS	FO	5.69 ± 0.38		
	MUFA	5.04 ± 0.19	D x S	P = 0.18
	N6:N3	5.86 ± 0.30		

Values are means and SEM, (n=6 for F1B, n=6 for GS)
Groups were compared by two-way ANOVA, $p<0.05$
D, S represent diet and strain respectively

Figure 3.1: F_1B hamsters fed the fish oil diet had milky plasma containing chylomicron-like particles. F_1B hamsters were fed the monounsaturated fatty acid-rich (MUFA) or fish oil (FO) diet for four weeks. Blood was collected via cardiac puncture and separated by centrifugation at 3000rpm for ten minutes.

MUFA FO

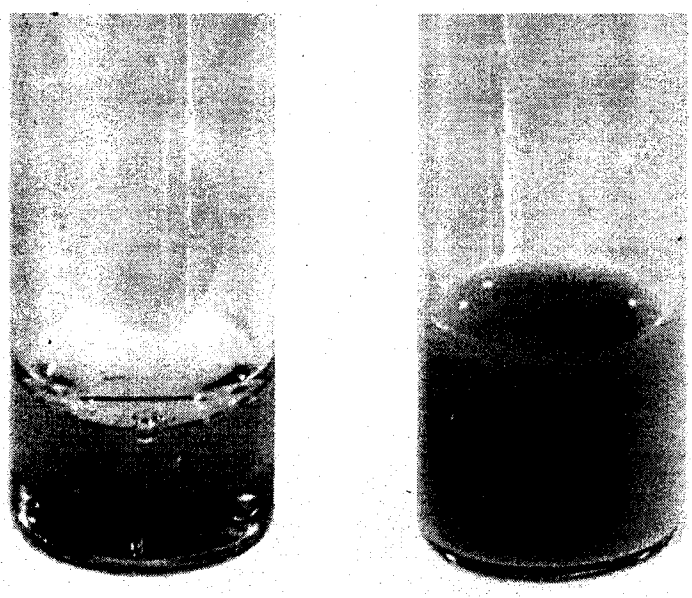


Figure 3.2: Plasma total cholesterol concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected and analyzed for total cholesterol as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. Values with unlike lower case letters are statistically significant.

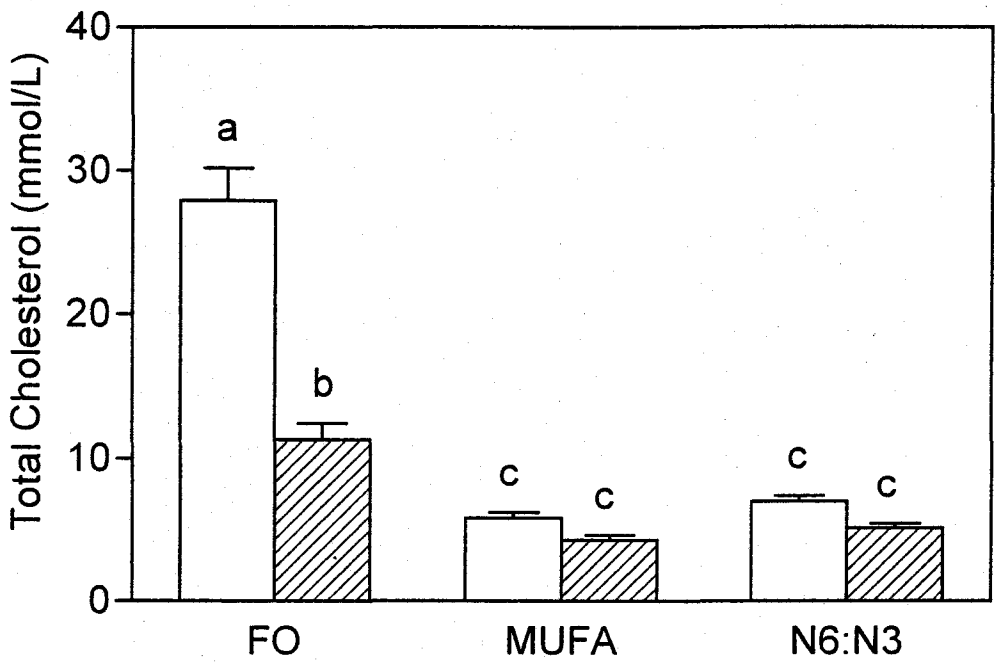
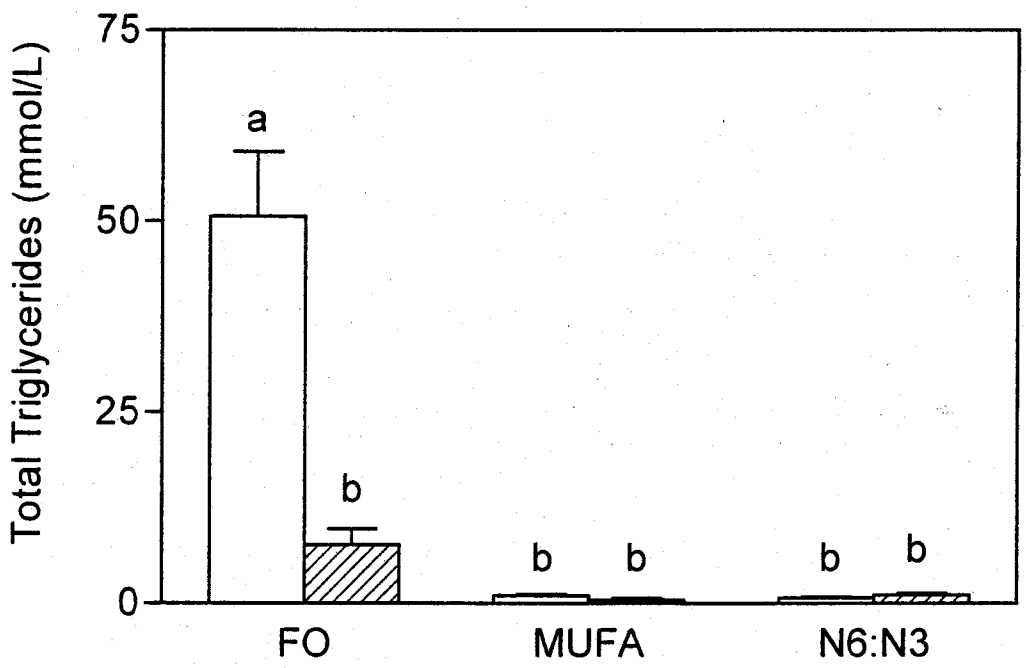

Total Cholesterol (mmol/L)
40
30
20
10
0
a
b
c
c
c
c
FO
MUFA
N6:N3

Figure 3.3: Plasma triglyceride concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected and analyzed for triglycerides as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. Values with unlike lower case letters are statistically significant.


Total Triglycerides (mmol/L)
75
50
25
0
a
b
b
b
b
b
FO
MUFA
N6:N3

hamsters on the fish oil diet were 3 times that of fish oil fed GS hamsters. There was a trend for an increase in plasma cholesterol concentrations in F_1B hamsters on the MUFA and N6:N3 diets compared to GS hamsters, however this increase did not reach statistical significance. There were no significant differences between the MUFA and N6:N3 diets on plasma cholesterol concentrations in F_1B or GS hamsters.

Plasma TG concentrations display a similar trend to plasma cholesterol concentrations (Figure 3.3). Fasting plasma TG concentrations in fish oil fed F_1B hamsters were markedly higher ($P < 0.0001$) than F_1B hamsters fed the MUFA and N6:N3 diets. In contrast, there was no significant effect of fish oil feeding on total plasma TG concentrations in GS hamsters as compared to GS hamsters on the MUFA and N6:N3 diets. Plasma TG concentrations in fish oil fed F_1B hamsters were 5 times higher than fish oil fed GS hamsters ($P < 0.0001$). There was no significant difference between the MUFA and N6:N3 diets on plasma TG concentrations in either F_1B or GS hamsters.

Plasma FC (Figure 3.4) and CE (Figure 3.5) concentrations show a similar trend as the plasma cholesterol and TG concentrations. One-way ANOVA revealed significantly higher plasma FC concentrations in fish oil fed F_1B hamsters compared to the MUFA and N6:N3 diets ($P < 0.001$). Fish oil feeding to GS hamsters also increased plasma FC concentrations compared to the MUFA and N6:N3 diets ($P < 0.01$). Similar to plasma cholesterol and TG concentrations, fish oil fed F_1B hamsters had significantly higher plasma FC concentrations than GS hamsters ($P < 0.001$). There were no significant effects of MUFA or N6:N3 feeding on plasma FC concentrations in either F_1B or GS hamsters.

Figure 3.4: Plasma free cholesterol concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected and analyzed for free cholesterol as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. Values with unlike lower case letters are statistically significant.

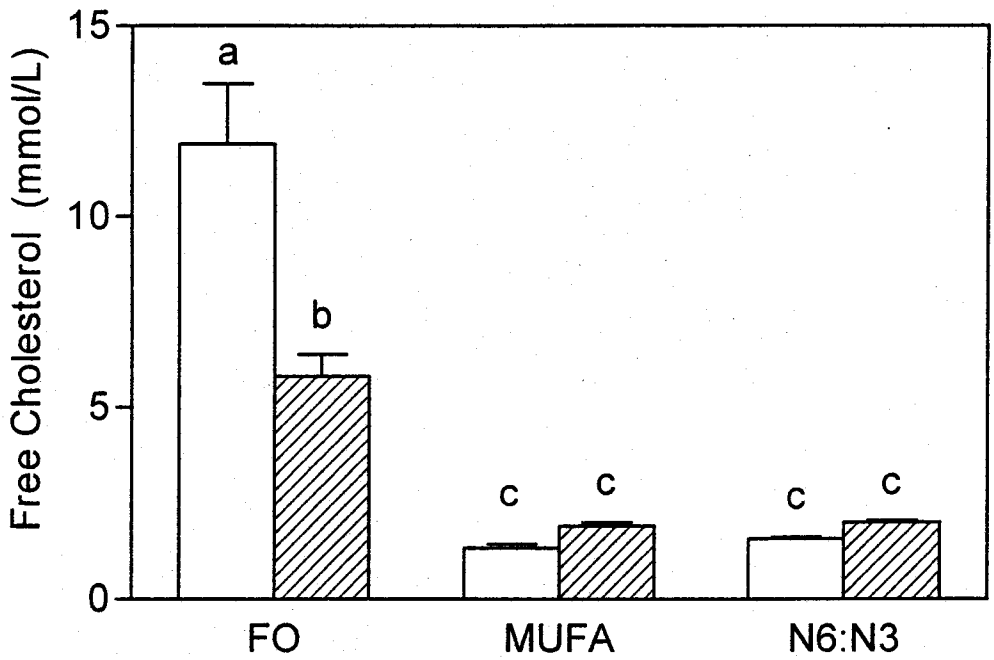
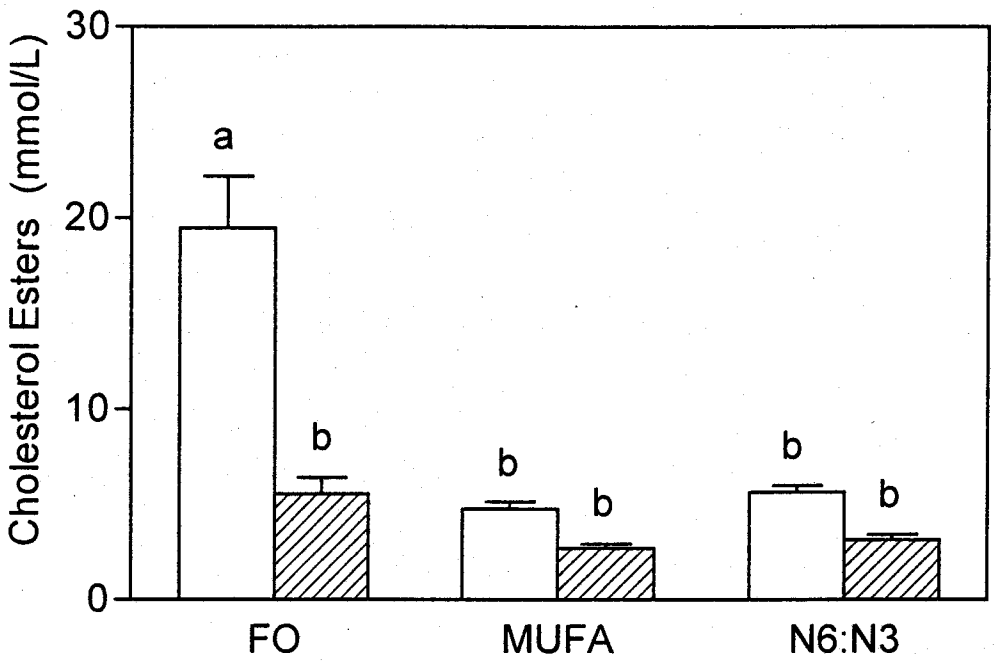

Free Cholesterol (mmol/L)
15
10
5
0
a
b
c
c
c
c
FO
MUFA
N6:N3

Figure 3.5: Plasma cholesterol ester concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected and analyzed for cholesterol esters as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. Values with unlike lower case letters are statistically significant.


Cholesterol Esters (mmol/L)
30
20
10
0
a
b
b
b
b
b
FO
MUFA
N6:N3

Analysis of plasma CE concentrations revealed that fish oil fed F_1B hamsters had significantly higher concentrations than F_1B hamsters fed the MUFA or N6:N3 diets ($P < 0.001$) (Figure 3.5). In contrast, fish oil feeding did not significantly increase CE concentrations in GS hamsters compared to the MUFA and N6:N3 diets ($P > 0.05$). There were also no significant effects of MUFA or N6:N3 feeding on plasma CE concentrations in F_1B or GS hamsters.

3.3 FPLC Profile

Fasting plasma samples from both F_1B and GS hamsters on all three diets were separated on a superose column via FPLC (Figure 3.6). While chylomicrons were separated before FPLC analysis, the VLDL fraction should be considered to be VLDL and chylomicrons as complete removal of chylomicrons from the sample was not possible. F_1B hamsters fed the fish oil diet had a significant portion of their plasma cholesterol present in the VLDL fraction in comparison to those hamsters on the MUFA and N6:N3 diets (Figure 3.6A). Fish oil fed GS hamsters also had higher VLDL-cholesterol compared to GS hamsters on the MUFA and N6:N3 diets (Figure 3.6B). This increase, was dramatically higher for F_1B hamsters. Both F_1B and GS hamsters on the MUFA and N6:N3 diets had a comparable amount of their plasma cholesterol in the VLDL, LDL, and HDL fractions.

3.4 Plasma VLDL Profile

Since the FPLC profile revealed dramatic differences in individual lipoprotein fractions between diet and hamster strain, plasma lipoprotein fractions were separated by ultra-centrifugation and analysed for cholesterol, TG, FC and CE concentrations. There

Figure 3.6: FPLC separation of fasted plasma samples from F_1B (A) and Golden Syrian (B) hamsters fed the fish oil (solid line) (–) monounsaturated fatty acid-rich (MUFA) (dashed line) (---), and N6:N3 (dotted line) (···) diets. Plasma samples were pooled from three animals in each group, filtered and analyzed on a Superose column as described in the materials and methods section.

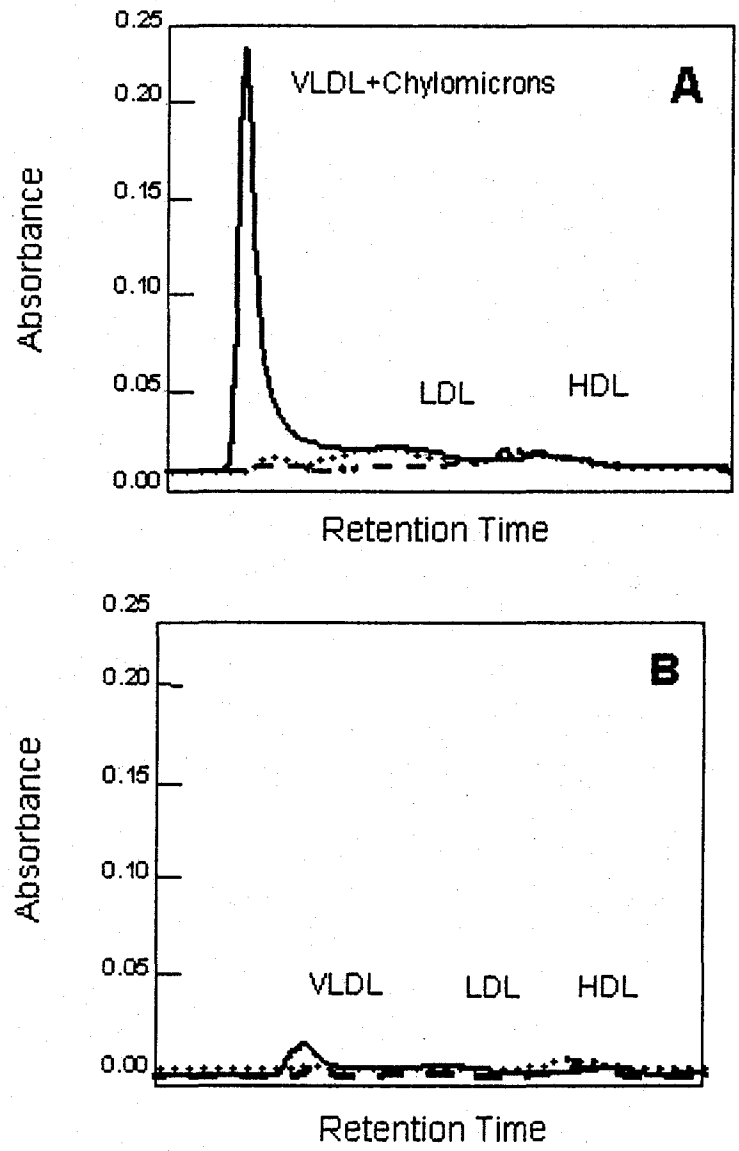

0.25
0.20
0.15
0.10
0.05
0.00
Absorbance
VLDL+Chylomicrons
A
LDL
HDL
Retention Time
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance
B
VLDL
LDL
HDL
Retention Time

Figure 3.7: Plasma very-low-density lipoprotein (VLDL) cholesterol concentrations in F_1B (☐) and Golden Syrian hamsters (▨) fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected and VLDL was separated by density gradient ultracentrifugation and analyzed for total cholesterol as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.

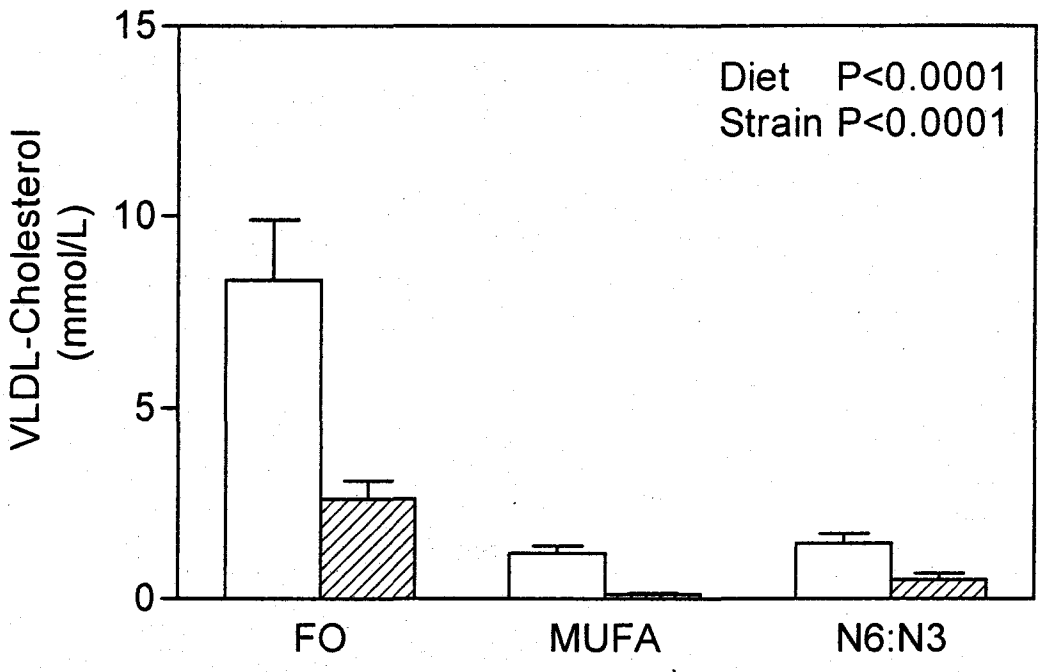

15
10
5
0
VLDL-Cholesterol (mmol/L)
Diet P<0.0001
Strain P<0.0001
FO
MUFA
N6:N3

Figure 3.8: Plasma very-low-density lipoprotein (VLDL) triglyceride concentrations in F_1B (□) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected, VLDL was separated by density gradient ultracentrifugation, and analyzed for tirglycerides as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.

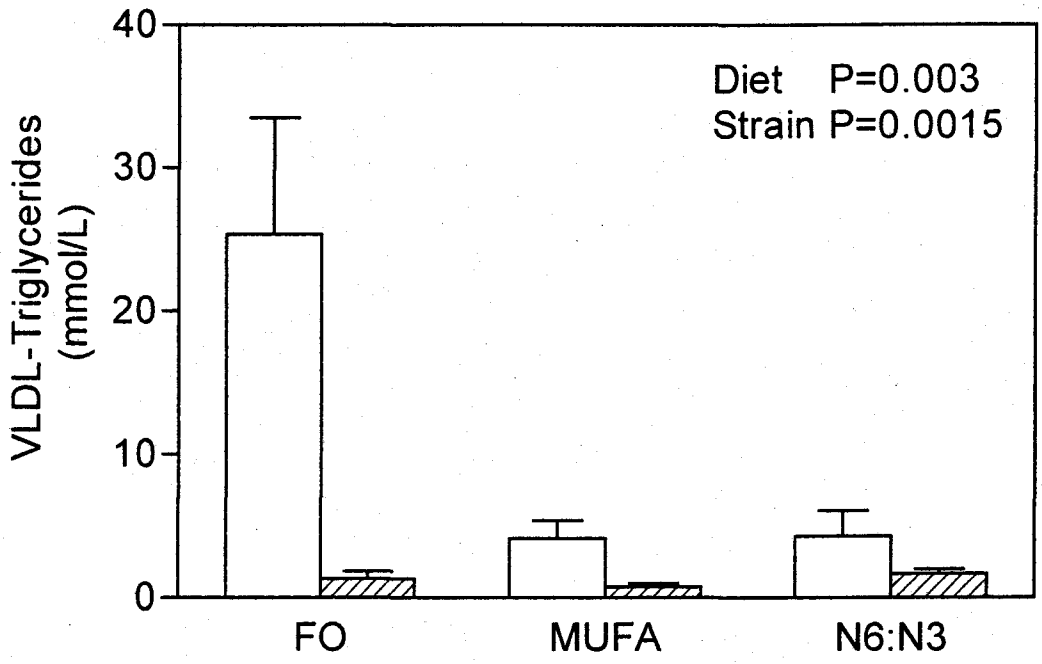

40
30
20
10
0
VLDL-Triglycerides (mmol/L)
Diet P=0.003
Strain P=0.0015
FO
MUFA
N6:N3

Figure 3.9: Plasma very-low-density lipoprotein (VLDL) free cholesterol concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected, VLDL was separated by density gradient ultracentrifugation, and analyzed for free cholesterol as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.

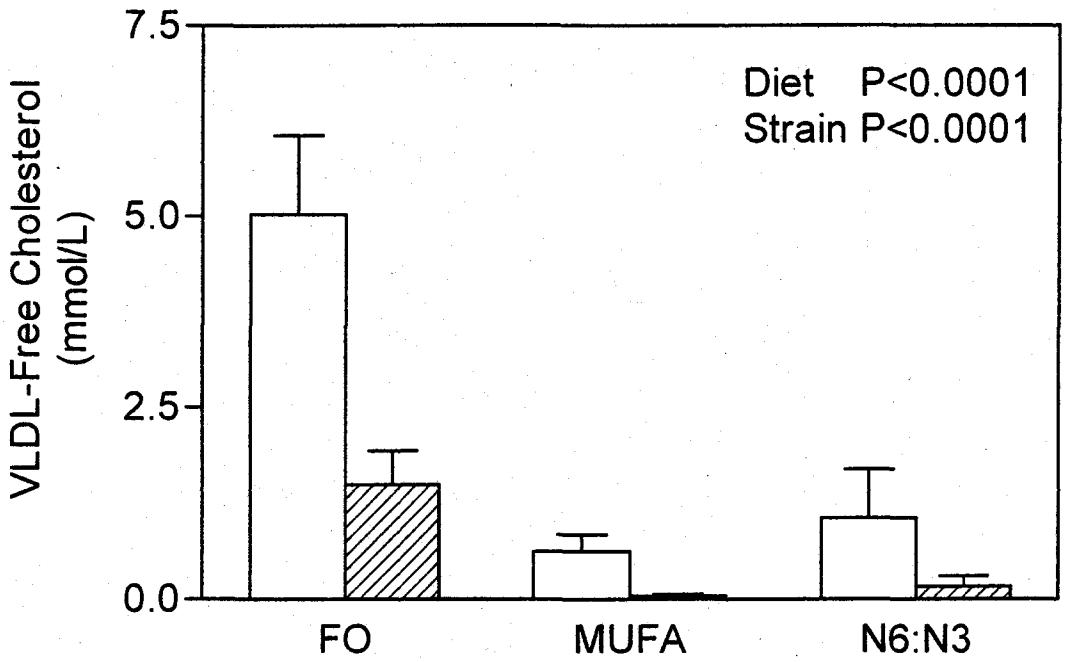
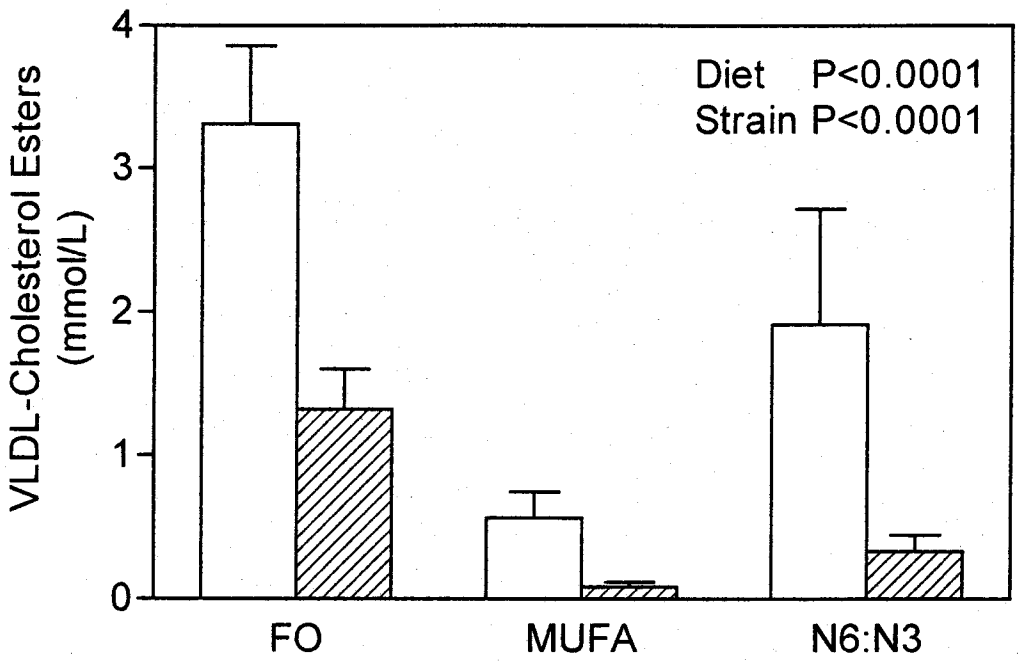

VLDL-Free Cholesterol (mmol/L)
7.5
5.0
2.5
0.0
Diet P<0.0001
Strain P<0.0001
FO
MUFA
N6:N3

Figure 3.10: Plasma very-low-density lipoprotein (VLDL) cholesterol ester concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected, VLDL was separated by density gradient ultracentrifugation, and analyzed for cholesterol esters as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.


VLDL-Cholesterol Esters
(mmol/L)
4
3
2
1
0
Diet P<0.0001
Strain P<0.0001
FO
MUFA
N6:N3

was a marked difference in VLDL lipid composition between F_1B and GS hamsters (Figures 3.7-3.10). VLDL-cholesterol, - TG, -FC, and -CE were all influenced by both diet ($P < 0.0001$, $P = 0.0014$, $P < 0.0001$, and $P < 0.0001$ respectively) and animal strain ($P < 0.0001$, $P = 0.0003$, $P < 0.0001$, and $P < 0.0001$ respectively) with a significant interaction between diet and strain for VLDL-cholesterol ($P = 0.0007$), -TG ($P = 0.0015$) and –FC concentrations ($P < 0.0001$).

Fish oil feeding to F_1B hamsters was associated with a dramatic increase in VLDL-cholesterol, - TG, -FC, and -CE concentrations in comparison to the MUFA and N6:N3 diets (figures 3.7-3.10 respectively). In GS hamsters however, fish oil feeding did not have as significant an impact on VLDL lipid parameters as was seen in F_1B hamsters. VLDL-cholesterol concentrations in fish oil fed F_1B hamsters were twice those seen in fish oil fed GS hamsters, while the VLDL-TG concentrations in fish oil fed F_1B hamsters were 25 times that of the GS hamsters. VLDL-FC and -CE concentrations in fish oil fed F_1B hamsters are also elevated compared to GS hamsters on the fish oil diet.

Due to the nature of the statistical analysis and the large influence of fish oil on VLDL lipids, the effects of the MUFA and N6:N3 diets on VLDL lipid parameters are not statistically significant. However, there does appear to be a trend for an increase in VLDL-cholesterol, -TG, -FC, and -CE in F_1B and GS hamsters on the N6:N3 diet compared to both hamster strains on the MUFA diet.

3.4.1 Plasma LDL Profile

Figures 3.11-3.14 depict the LDL lipid profile for F_1B and GS hamsters. LDL-cholesterol, -TG, -FC, and -CE were all independently influenced by both diet ($P =$

0.0006, $P < 0.0001$, $P < 0.0001$, and $P = 0.001$ respectively), and animal strain ($P = 0.0004$, $P = 0.0348$, $P = 0.0005$, and $P = 0.0003$ respectively). Only LDL-TG however showed an interactive effect of diet and animal strain ($P = 0.0118$).

Figure 3.11: Plasma low-density lipoprotein (LDL) cholesterol concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected, LDL was separated by density gradient ultracentrifugation, and analyzed for total cholesterol as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.

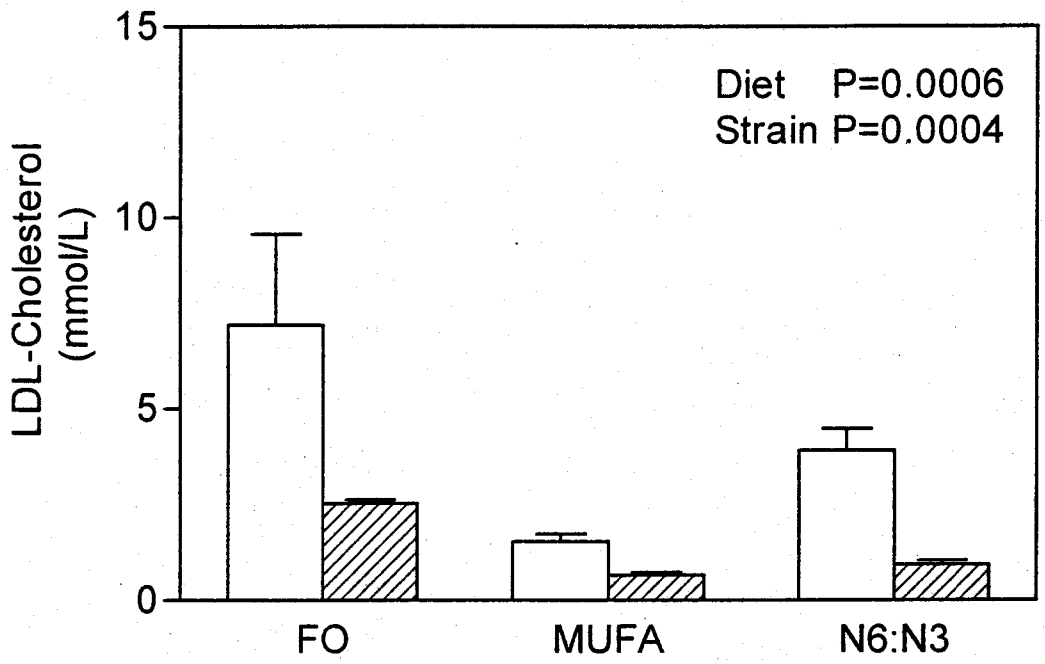

15
10
5
0
LDL-Cholesterol (mmol/L)
Diet P=0.0006
Strain P=0.0004
FO
MUFA
N6:N3

Figure 3.12: Plasma low-density lipoprotein (LDL) triglyceride concentrations in F_1B (□) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected, LDL was separated by density gradient ultracentrifugation, and analyzed for triglycerides as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.

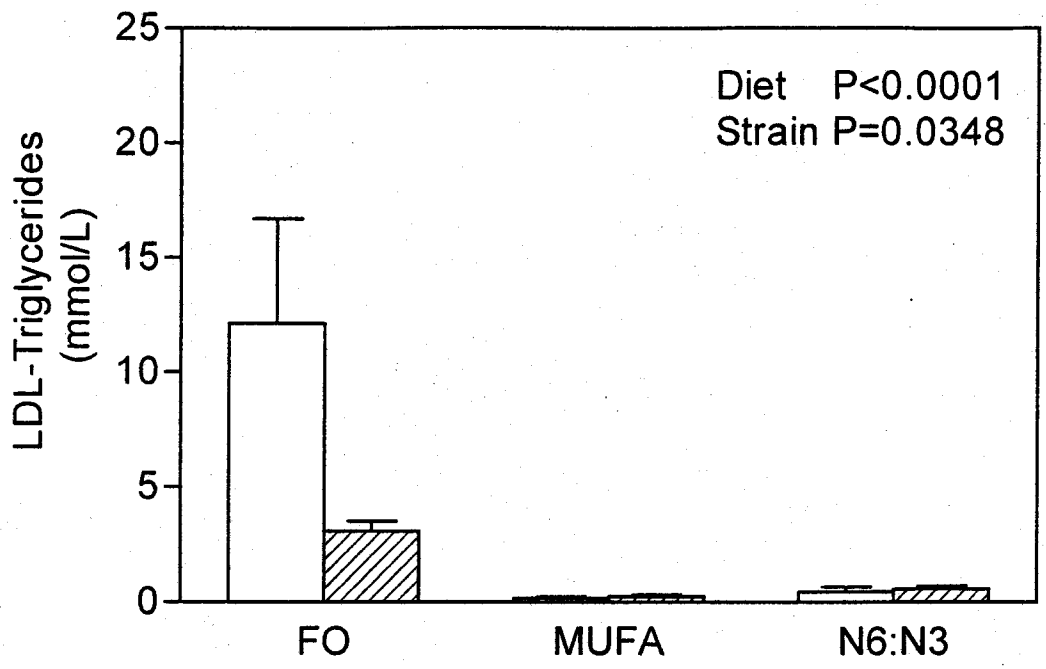

LDL-Triglycerides (mmol/L)
25
20
15
10
5
0
Diet P<0.0001
Strain P=0.0348
FO
MUFA
N6:N3

Figure 3.13: Plasma low-density lipoprotein (LDL) free cholesterol concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected, LDL was separated by density gradient ultracentrifugation, and analyzed for free cholesterol as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.

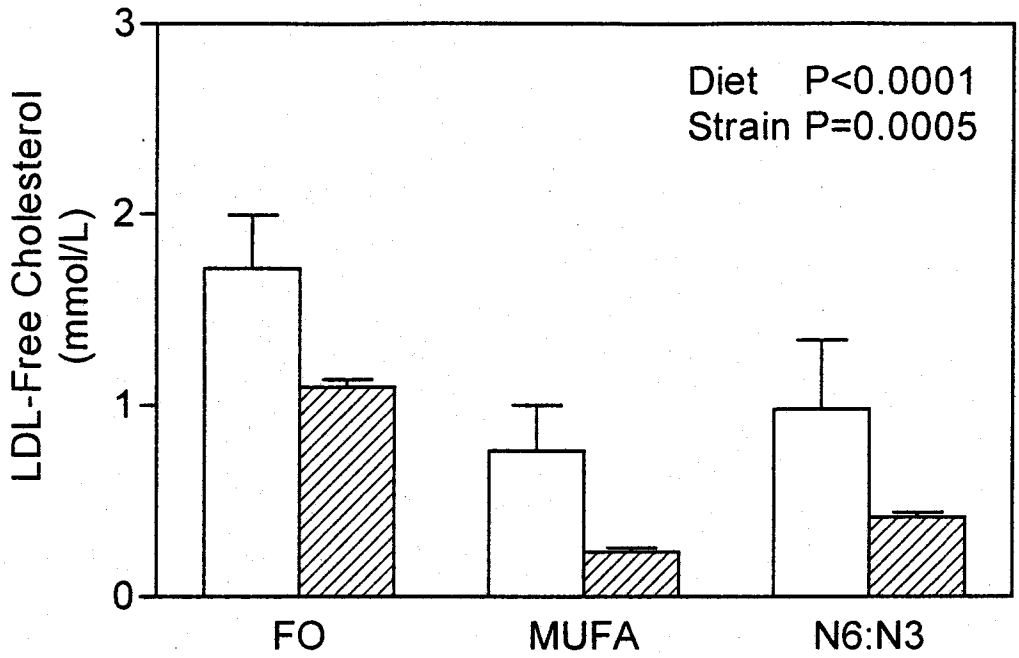
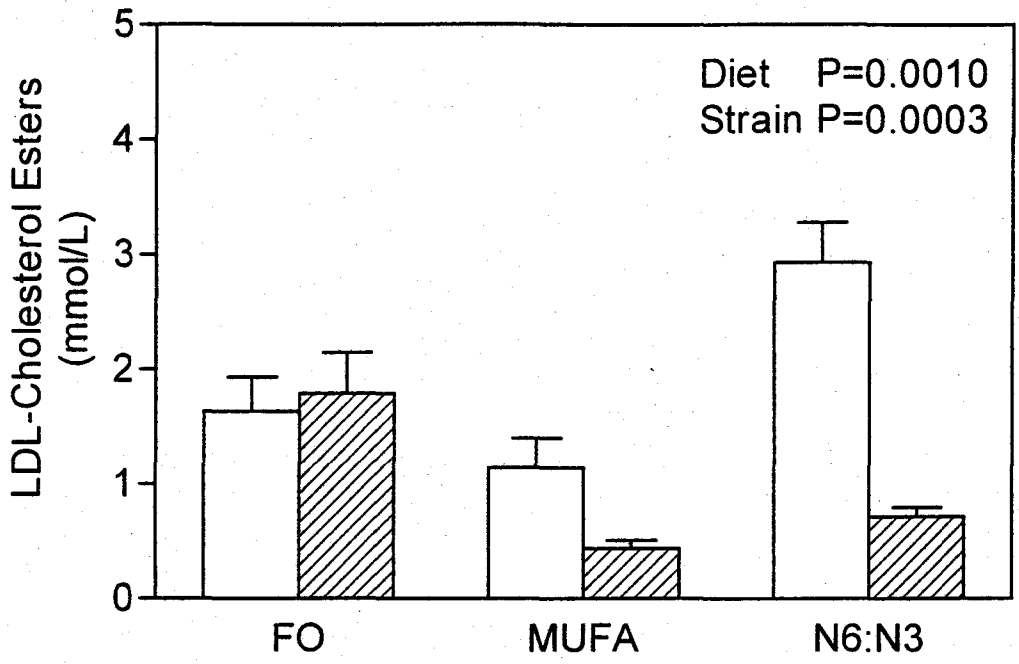

LDL-Free Cholesterol (mmol/L)
3
2
1
0
Diet P<0.0001
Strain P=0.0005
FO
MUFA
N6:N3

Figure 3.14: Plasma low-density lipoprotein (LDL) cholesterol ester concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected, LDL was separated by density gradient ultracentrifugation, and analyzed for cholesterol esters as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.


LDL-Cholesterol Esters
(mmol/L)
5
4
3
2
1
0
Diet P=0.0010
Strain P=0.0003
FO
MUFA
N6:N3

Fish oil feeding was associated with an increase in LDL-cholesterol, -TG, and -FC concentrations in F_1B hamsters compared to the F_1B hamsters fed the MUFA or N6:N3 diet. Fish oil-fed GS hamsters also had elevated levels of LDL-cholesterol, -TG, and -FC concentrations compared to those on the MUFA and N6:N3 diets. Comparison of the two strains shows that this increase was much more pronounced in F_1B than GS hamsters. LDL-cholesterol and TG concentrations in fish oil fed F_1B hamsters were approximately two times higher than those seen in fish oil fed GS hamsters.

Interestingly, F_1B hamsters fed the N6:N3 diet show a trend toward an increase in LDL-cholesterol, and -CE concentrations compared to MUFA-fed hamsters. The LDL-cholesterol and -CE concentrations in F_1B hamsters on the N6:N3 diet were two-fold higher than F_1B hamsters fed the MUFA diet.

3.4.2 Plasma HDL profile

Figures 3.15-3.18 depict the HDL-cholesterol, -TG, -FC, and -CE concentrations respectively in F_1B and GS hamsters. Diet had a significant influence on HDL-TG ($P = 0.0035$), -FC ($P = 0.0102$), and -CE ($P = 0.0035$) concentrations, while strain independently influenced HDL-cholesterol ($P = 0.0163$) and -FC ($P = 0.0120$) concentrations. The only interactive effect between diet and strain was observed for HDL-CE concentrations ($P = 0.0129$).

There was no effect of diet on HDL-cholesterol concentrations in F_1B or GS hamsters. Fish oil feeding to F_1B and GS hamsters however, largely influenced HDL-TG and -FC concentrations compared to the MUFA and N6:N3 diets. Fish oil-fed F_1B hamsters had HDL-TG concentrations that were three times those seen in fish oil fed GS

Figure 3.15: Plasma high-density lipoprotein (HDL) cholesterol concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected, HDL was separated by density gradient ultracentrifugation, and analyzed for total cholesterol as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.

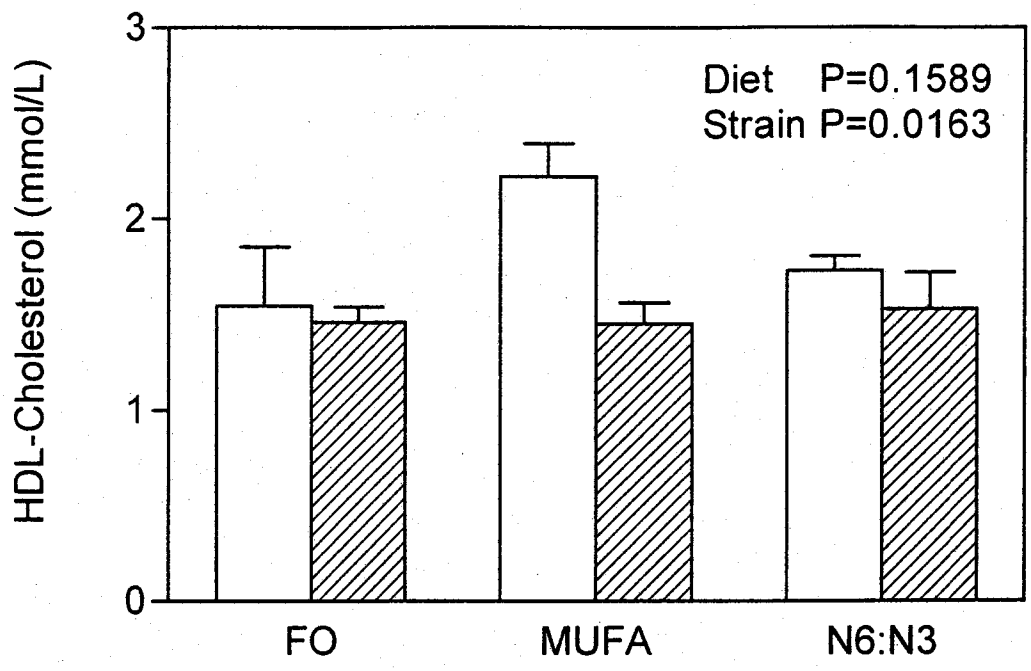

HDL-Cholesterol (mmol/L)
3
2
1
0
Diet P=0.1589
Strain P=0.0163
FO
MUFA
N6:N3

Figure 3.16: Plasma high-density lipoprotein (HDL) triglyceride concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected, HDL was separated by density gradient ultracentrifugation, and analyzed for triglycerides as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.

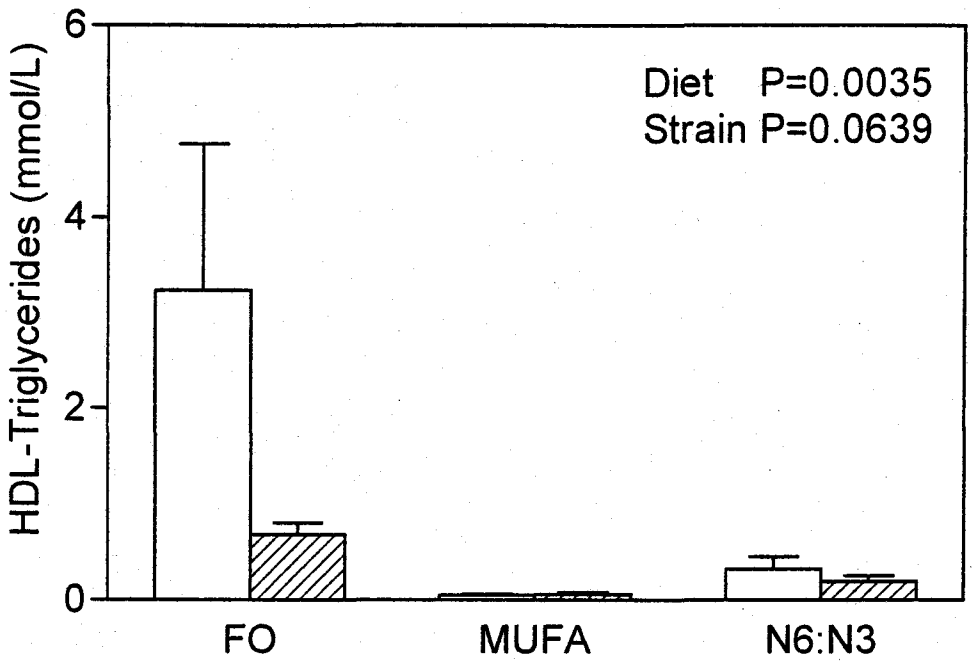

HDL-Triglycerides (mmol/L)
6
4
2
0
Diet P=0.0035
Strain P=0.0639
FO
MUFA
N6:N3

Figure 3.17: Plasma high-density lipoprotein (HDL) free cholesterol concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected, HDL was separated by density gradient ultracentrifugation, and analyzed for free cholesterol as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.

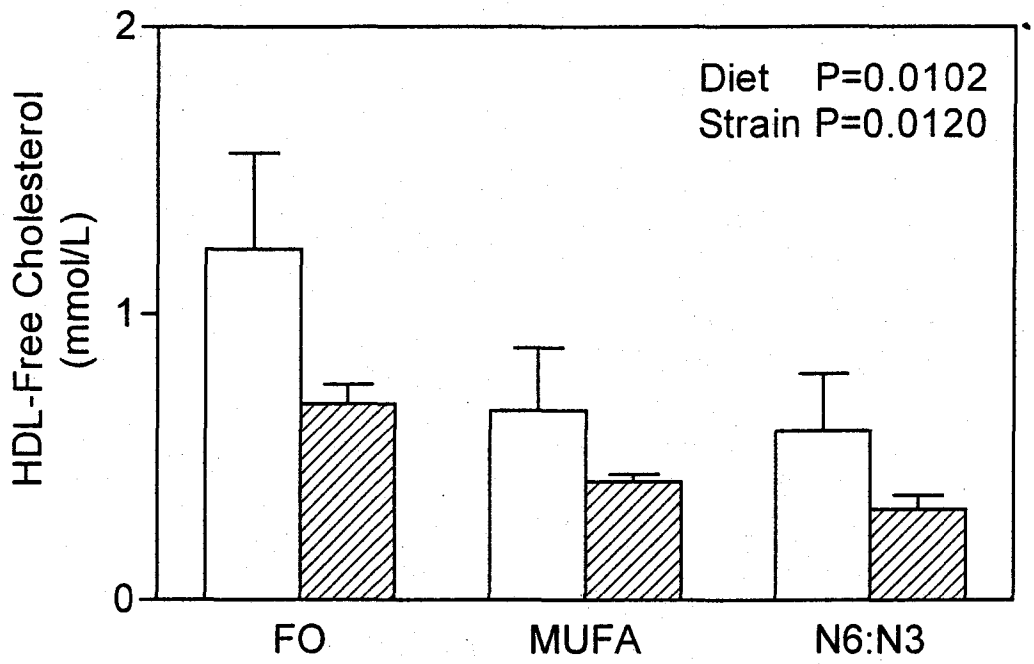
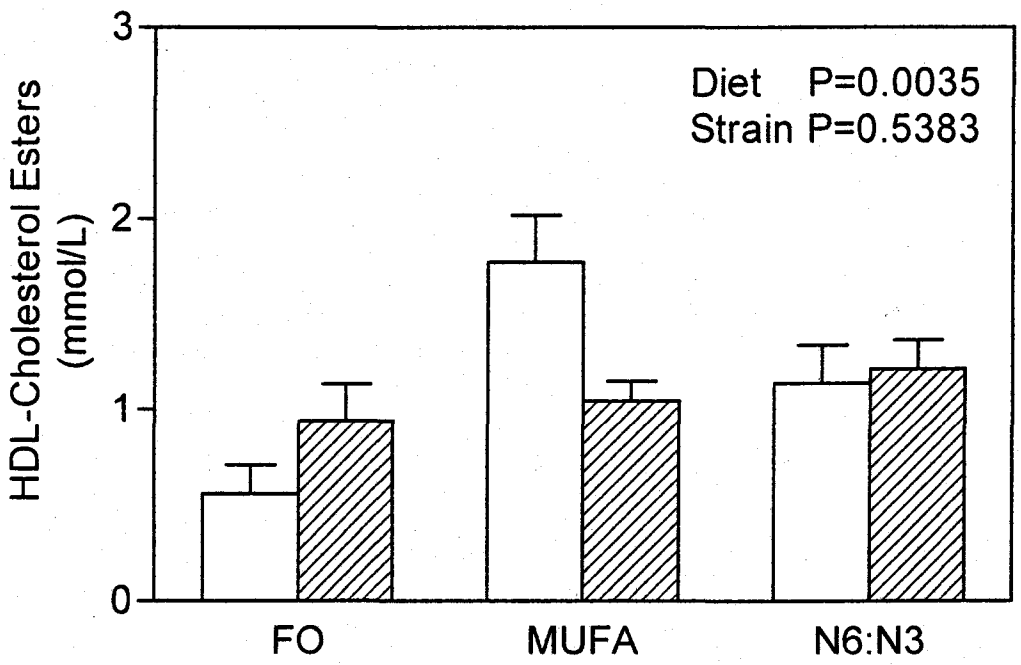

HDL-Free Cholesterol (mmol/L)
2
1
0
Diet P=0.0102
Strain P=0.0120
FO
MUFA
N6:N3

Figure 3.18: Plasma high-density lipoprotein (HDL) cholesterol ester concentrations in F_1B (☐) and Golden Syrian (▨) hamsters fed the fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diet for a period of 4 weeks. Fasting plasma samples were collected, HDL was separated by density gradient ultracentrifugation, and analyzed for cholesterol esters as described in the materials and method section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test. There was a significant interaction between diet and strain.


HDL-Cholesterol Esters
(mmol/L)
3
2
1
0
Diet P=0.0035
Strain P=0.5383
FO
MUFA
N6:N3

hamsters. Fish oil feeding to F_1B also raised HDL-FC concentrations compared to the MUFA and N6:N3 diets. Interestingly, F_1B and GS hamsters on the fish oil diet had lower HDL-CE concentrations than MUFA and N6:N3 fed F_1B and GS hamsters. This finding is in contrast to the results obtained for VLDL- and LDL-CE concentrations. Both HDL-cholesterol and -FC concentrations were also influenced by strain, where F_1B hamsters had consistently elevated HDL-cholesterol and -FC concentrations compared to GS hamsters.

Both F_1B and GS hamsters on the N6:N3 diet show a trend for an increase in HDL-TG concentrations compared to hamsters on the MUFA diet. This effect was not apparent however in HDL-FC or -CE concentrations.

3.5 Hepatic Lipid Profile

Hepatic lipid concentrations for F_1B and GS hamsters are given in Table 5. Hepatic total cholesterol, TG, and CE concentrations were significantly influenced by diet ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$ respectively). Fish oil feeding to F_1B and GS hamsters significantly elevated hepatic cholesterol, TG, and CE concentrations compared to F_1B and GS hamsters on the MUFA and N6:N3 diets. Hepatic FC concentrations were unaffected by hamster strain. The N6:N3 diet also elevated hepatic lipid concentrations in comparison to the MUFA diet. Liver total cholesterol and CE concentrations were approximately 1.5 times higher in F_1B and GS hamsters on the N6:N3 diet compared to the MUFA diet. Hepatic TG and FC concentrations remained similar in F_1B and GS hamsters on the MUFA and N6:N3 diets.

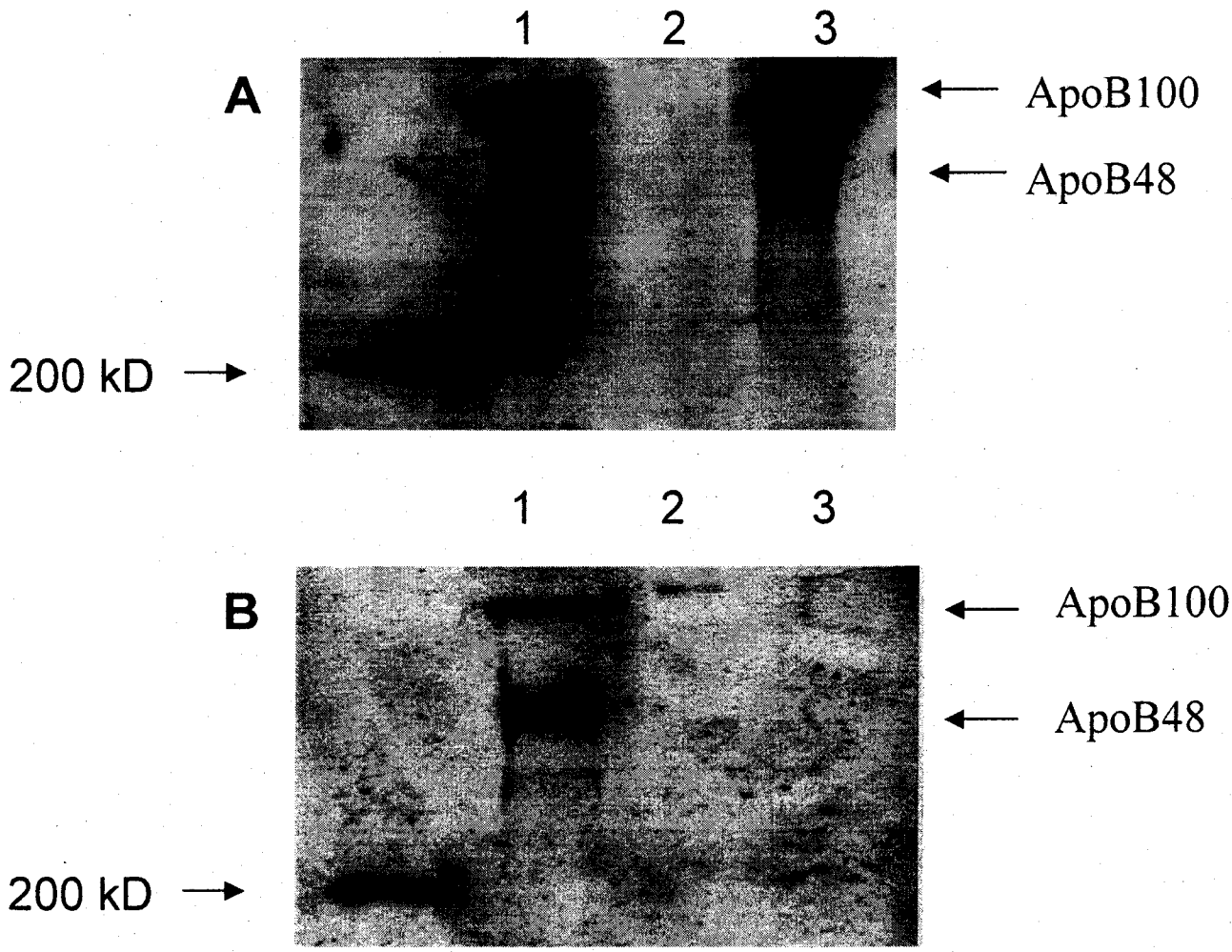
The hepatic TG concentration was the only parameter influenced by animal strain ($P < 0.0001$). Hepatic TG concentrations in GS hamsters on the fish oil, MUFA, and N6:N3 diets were two times higher than F_1B hamsters on the fish oil, MUFA, and N6:N3 diets.

3.6 Apolipoprotein B Protein Expression

Figure 3.19 depicts representative plasma protein expression of apoB100 and apoB48 in F_1B (A) and GS (B) hamsters on the fish oil (lane 1), MUFA (lane 2), and N6:N3 (lane 3) diets. F_1B hamsters on the fish oil diet had markedly higher plasma apoB100 protein expression than F_1B hamsters on the MUFA and N6:N3 diet. ApoB100 was undetectable in F_1B hamsters fed the MUFA diet at a protein concentration of 60μg. Surprisingly, after a 14-hour fast, plasma apoB48 was detected in F_1B hamsters on both the fish oil and N6:N3 diets. The expression of apoB48 in fish oil fed F_1B hamsters, however, was much greater than those hamsters on the N6:N3 diet. Fish oil feeding to GS hamsters had a similar effect as seen in F_1B hamsters. Both apoB100 and apoB48 were detectable in fish oil fed GS hamsters. ApoB100 was present in GS hamsters fed the MUFA and N6:N3 diets, but to a lesser extent than fish oil fed hamsters. ApoB48 was not detectable in the plasma of GS hamsters fed the MUFA or N6:N3 diets.

The expression of apoB100 and apoB48 differed greatly between hamster strains. Fish oil fed F_1B hamsters had markedly higher plasma protein expression of both apoB100 and apoB48 than GS hamsters on the fish oil diet. In addition, the presence of apoB48 as seen in F_1B hamsters fed the N6:N3 diet was not apparent in GS hamsters on the N6:N3 diet.

Figure 3.19: Apolipoprotein B protein expression in F_1B (A) and Golden Syrian (B) hamsters. Hamsters fed the fish oil, monounsaturated fatty-acid rich, and N6:N3 diets are represented in lanes 1, 2, and 3 respectively. Proteins were separated on a 6% polyacrylamide gel and transferred to a nitrocellulose membrane. Apolipoprotein B was detected by Western blotting as described in the materials and methods section. This figure is typical of all the samples analysed.


1
2
3
A
ApoB100
ApoB48
200 kD
1
2
3
B
ApoB100
ApoB48
200 kD

3.7 Microsomal Triglyceride Transfer Protein Activity

Figure 3.20 and figure 3.21 show the intestinal and hepatic MTTP activity respectively for F_1B and GS hamsters on all three diets. There was no significant effect of diet ($P = 0.15$) or strain ($P = 0.77$) on hepatic MTTP activity. Similarly, there was no significant effect of diet ($P = 0.74$) or strain ($P = 0.31$) on intestinal MTTP activity.

3.8 Lipoprotein Lipase Activity

Figure 3.22 depicts the post-heparin lipoprotein lipase activity in F_1B and GS hamsters on the fish oil, MUFA, and N6:N3 diets. There was a significant effect of strain ($P < 0.001$) on lipoprotein lipase activity, however there was no significant effect of either the fish oil, MUFA, or N6:N3 diet ($P = 0.26$). F_1B hamsters had markedly lower post-heparin lipoprotein lipase activity compared to GS hamsters on all three diets. While there was no significant effect of diet on post-heparin lipoprotein lipase activity, there is a trend for a decrease in LPL activity in F_1B hamsters fed the fish oil and N6:N3 diets.

3.9 Hepatic LDL-Receptor mRNA Expression

Figure 3.23 shows the hepatic LDL receptor mRNA expression in F1B and GS hamsters on all three diets. LDLr mRNA was normalized against hamster β-actin and expressed as relative units. There was no significant effect of either diet ($P = 0.10$) or strain ($P = 0.1676$) on LDLr mRNA expression.

Figure 3.20: Intestinal microsomal triglyceride transfer protein (MTTP) activity in F_1B (☐) and Golden Syrian (▨) hamsters fed fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diets. Animals were fed the specified diets for four weeks. Upon sacrifice, the intestines were removed and snap frozen in liquid nitrogen and stored at – 70°C. Tissues were then analyzed for MTTP activity as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test.

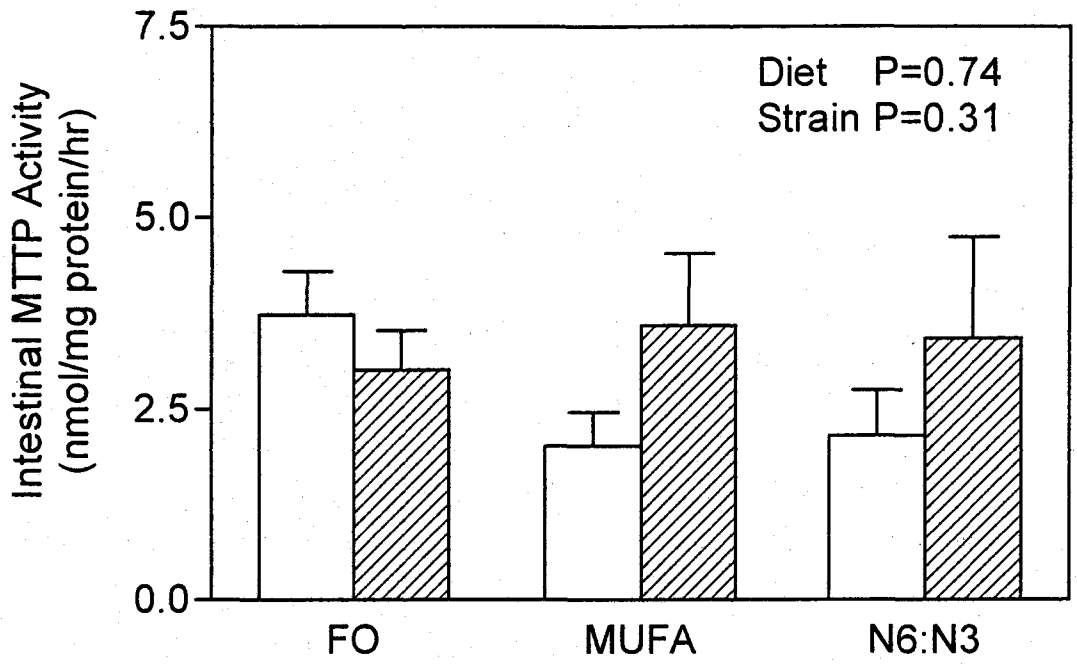

Intestinal MTTP Activity
(nmol/mg protein/hr)
7.5
5.0
2.5
0.0
Diet P=0.74
Strain P=0.31
FO
MUFA
N6:N3

Figure 3.21: Hepatic microsomal triglyceride transfer protein (MTTP) activity in F_1B (☐) and Golden Syrian (▨) hamsters fed fish oil (FO), monounsaturated fatty acid rich (MUFA) or N6:N3 diets. Animals were fed the specified diets for four weeks. Upon sacrifice, the livers were removed and snap frozen in liquid nitrogen and stored at –70°C. Tissues were then analyzed for MTTP activity as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test.

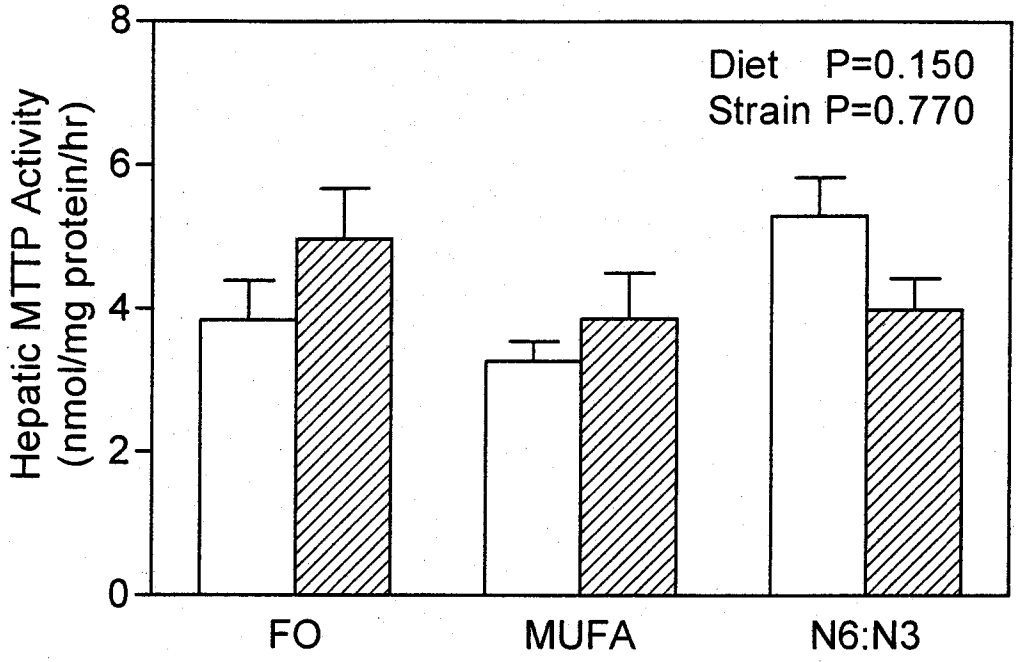

Hepatic MTTP Activity
(nmol/mg protein/hr)
8
6
4
2
0
Diet P=0.150
Strain P=0.770
FO
MUFA
N6:N3

Figure 3.22: Lipoprotein lipase activity in F_1B (☐) and Golden Syrian (▨) hamsters fed fish oil (FO), monounsaturated fatty acid rich (MUFA), and N6:N3 diets. The specified diets were fed for a period of four weeks. After a 14-hour fast, heparin was injected directly into the heart. Blood was collected and assayed for lipoprotein lipase activity as described in the materials and methods section. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test.

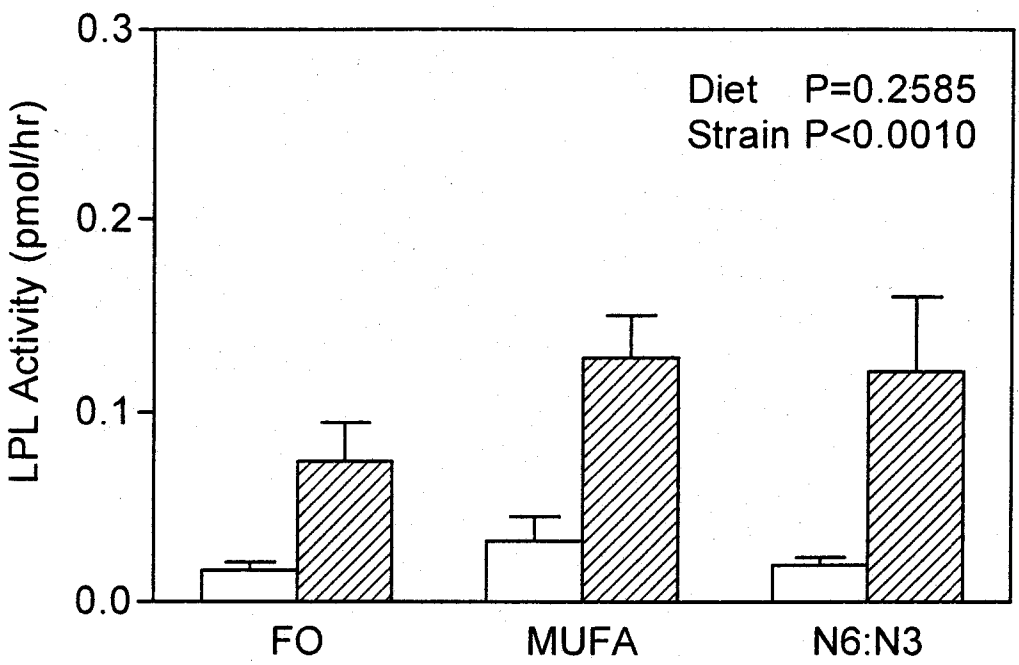
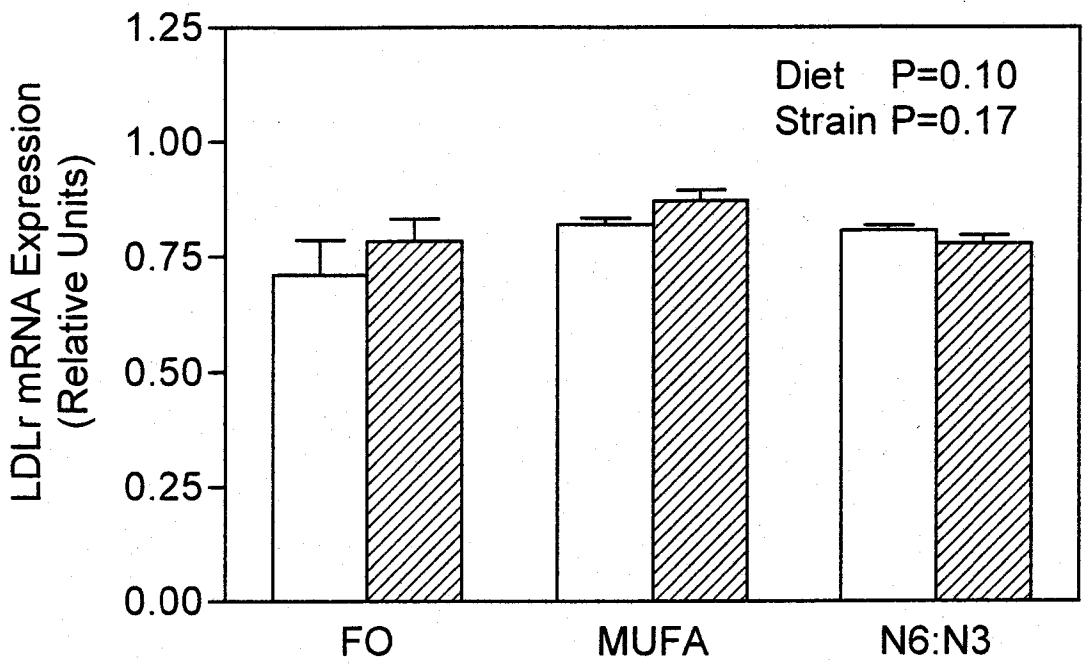

0.3
0.2
0.1
0.0
LPL Activity (pmol/hr)
Diet P=0.2585
Strain P<0.0010
FO
MUFA
N6:N3

Figure 3.23: LDL receptor mRNA expression in F1B (☐) and Golden Syrian (▨) hamsters. RT-PCR was performed on isolated hepatic RNA from both hamster strains on the fish oil, monounsaturated fatty acids rich (MUFA), and N6:N3 diets as described in the materials and methods section. LDLr mRNA expression was normalized to β-actin and expressed as relative units. Values are means and SEM (n = 6 F_1B; n = 6 GS) analyzed by two-way ANOVA and the Newman-Keuls post-hoc test.


LDLr mRNA Expression
(Relative Units)
1.25
1.00
0.75
0.50
0.25
0.00
Diet P=0.10
Strain P=0.17
FO
MUFA
N6:N3

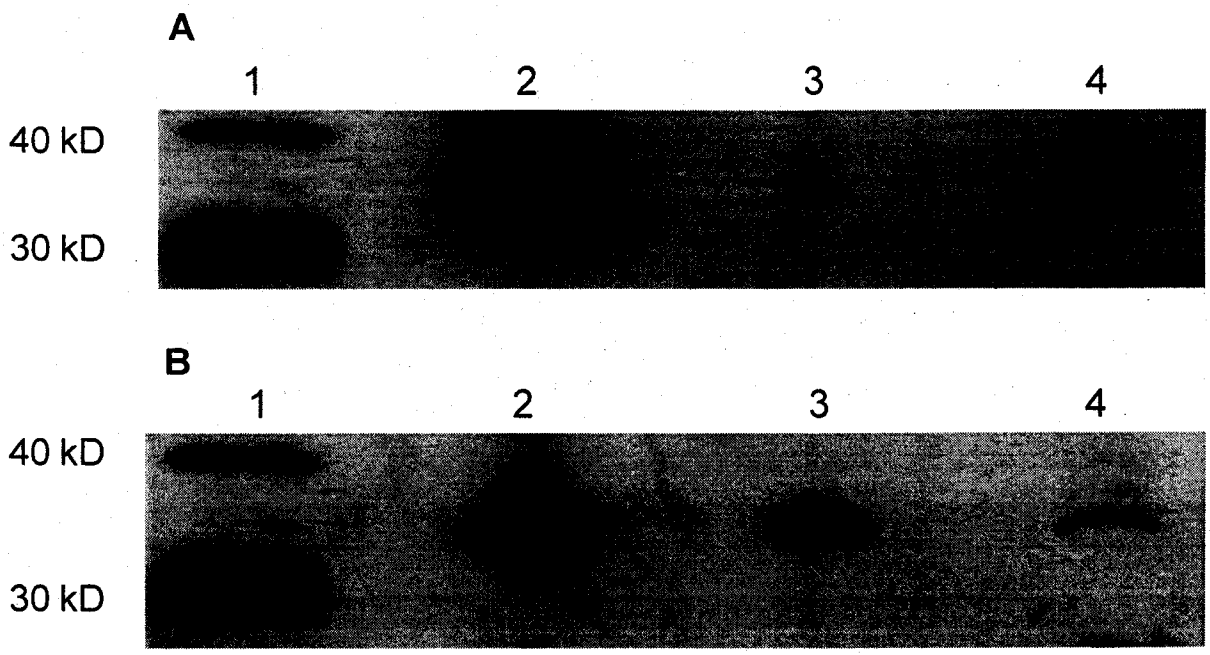
3.10 Plasma Apolipoprotein E Protein Expression

Figure 3.24 depicts the plasma ApoE protein expression in F_1B (A) and GS (B) hamsters on the fish oil (lane 2), MUFA (lane 3), and N6:N3 (lane 4) diets. All three diets had varying effects on plasma apoE protein expression in F_1B and GS hamsters. F_1B hamsters on the fish oil diet had markedly higher plasma apoE expression than F_1B hamsters on the MUFA and N6:N3 diets. In addition, MUFA fed F_1B hamsters had the least expression of apoE. F_1B hamsters fed the N6:N3 diet had much higher expression of plasma apoE than those fed the MUFA diet.

Fish oil-fed GS hamsters had markedly higher plasma apoE expression than GS hamsters on the MUFA and N6:N3 diets, similar to the findings for the F_1B hamsters. In contrast however, GS hamsters on the MUFA diet had an increase in apoE protein expression compared to those hamsters on the N6:N3 diet.

There is an apparent difference in apoE expression between F_1B and GS hamsters on all three diets. F_1B hamsters on the fish oil diet have an increase in apoE protein expression compared to fish oil fed GS hamsters. In addition, the effects of the MUFA and N6:N3 diets in F_1B hamsters are reversed in GS hamsters. MUFA fed F_1B hamsters show a decrease in apoE protein expression compared to MUFA fed GS hamsters, while F_1B hamsters on the N6:N3 diet have markedly higher apoE expression than GS hamsters on the N6:N3 diets.

Figure 3.24: Apolipoprotein E protein expression in the plasma of F_1B (A) and Golden Syrian (B) hamsters. The position of the molecular weight markers are indicated in lane 1. Hamsters fed the fish oil, monounsaturated fatty acid rich, and N6:N3 diets are represented in lanes 2, 3, and 4 respectively. Plasma proteins were separated on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. ApoE proteins were detected by Western blotting as described in the materials and methods section. This figure is typical of all (n = 8) samples analyzed.


A
1
2
3
4
40 kD
30 kD
B
1
2
3
4
40 kD
30 kD

Chapter 4:

Discussion

4.1 Dietary Fats and CVD

Many studies have shown that the quantity and quality of dietary fats can regulate lipid and lipoprotein metabolism, thus influencing the development of CVD. While SFA are known to promote hyperlipidemia and CVD development, PUFA, particularly the n-3 fatty acids found in fish oil, are known to improve the plasma lipid profile and decrease the risk of CVD (Harris, 1989). Fish oil exerts its cardioprotective properties by suppressing plasma TG concentrations in a dose-dependent manner (Wong *et al.*, 1984; Harris, 1989). There is however, a controversy that surrounds the recommendation of fish oil as an anti-atherogenic therapy. The effects of fish oil on plasma and LDL-cholesterol concentrations are inconsistent. Several investigators have found that fish oil feeding to humans and animals can significantly raise plasma and LDL-cholesterol concentrations, which are a known risk factor for CVD (Dewailly, *et al.*, 2001).

Previous work in our lab has examined the regulation of lipid and lipoprotein metabolism by fish oil (de Silva *et al.*, 2004). F_1B hamsters were fed a fish oil diet for a period of two weeks. Surprisingly, these hamsters developed severe dyslipidemia with highly elevated plasma-, VLDL-, and LDL-cholesterol and TG concentrations, and the presence of milky plasma after a 14-hour fast. This unexpected response led us to believe that fish oil was affecting either the synthesis and secretion, or the clearance of TG- and cholesterol-rich lipoproteins. Thus in this study, we used three cardioprotective diets rich in either fish oil, MUFA, or containing an optimal n-6:n-3 ratio in F_1B and GS hamsters. The use of these three diets allows us to compare the response of F_1B and GS hamsters to other dietary fatty acids to determine if the previously found dyslipidemia is specific to fish oil. In addition, the fish oil-induced elevation in LDL-cholesterol concentrations

previously observed in F_1B hamsters affords a unique opportunity to examine the mechanisms by which fish oil may be increasing plasma cholesterol concentrations.

There is a great deal of heterogeneity in genetic background amongst humans, which is partially responsible for the varied responses to dietary fatty acid supplementation. Therefore, in this study, the comparison of F_1B and GS hamsters allows us to determine if the response to fish oil is strain specific. The existence of polymorphisms between animal strain may be responsible for the fish oil induced hyperlipidemia in F_1B hamsters, and may provide insight into the differential response to fatty acid supplementation in humans.

4.1.1 Fish Oil and the Plasma Lipid Profile

Fish oil is considered to be cardioprotective as it exerts beneficial effects on the plasma lipid profile by lowering plasma TG concentrations compared to other dietary fats. In this study however, fish oil feeding induced hyperlipidemia in both F_1B and GS hamsters with elevations in plasma cholesterol, TG, FC, and CE concentrations compared to the MUFA and N6:N3 diets. F_1B hamsters, in particular, had milky plasma after a 14-hour fast when fed the fish oil diet, suggesting an inhibitory effect of fish oil on lipoprotein clearance. This study, and previously published work in our lab (de Silva *et al.*, 2004) are the first to demonstrate the hyperlipidemic effect of fish oil in F_1B hamsters. The dyslipidemic effect of fish oil has however, been reported by other investigators in GS hamsters (Surette *et al.*, 1992; Lin *et al.*, 1995; Kubow *et al.*, 1996; Lu *et al.*, 1996). GS hamsters receiving a diet with Menhaden oil and cholesterol for 27 days for example, had significantly higher serum cholesterol and TG concentrations than hamsters on a safflower oil diet (Kubow *et al.*, 1996). Elevated TG concentrations have

also previously been reported in hamsters and New Zealand rabbits consuming diets containing fish oil (Hayes *et al.*, 1992). Similarly, fish oil feeding to GS hamsters was shown to significantly increase plasma cholesterol concentrations in the presence of dietary cholesterol (Lin *et al.*, 1995; Lu *et al.*, 1996). Interestingly, in the absence of cholesterol, these authors found that fish oil supplementation decreased plasma cholesterol and TG concentrations.

In humans, fish oil feeding has consistently been shown to reduce TG concentrations, primarily by reducing apoB and VLDL synthesis and secretion. There are several reports however, which have demonstrated that fish oil increases plasma cholesterol concentrations, mainly in the LDL fraction (Harris, 1989; Wilt *et al.*, 1989). The hyperlipidemic effect of fish oil in this study, denoted by milky plasma in F_1B hamsters and highly elevated plasma TG concentrations, may be attributed to an increase in the production of TG-rich lipoproteins, or a reduction in their clearance.

There are several factors related to the fatty acid composition of the fish oil diet which should be taken into consideration. The cholesterol content of the diet (0.25%) is relatively high. F_1B hamsters have been shown to respond to dietary cholesterol with large increases in plasma TG and cholesterol concentrations. Therefore, while all animals were given diets with the same cholesterol content, it is possible that the vast variations plasma lipid concentrations are due to the MUFA and N6:N3 diets reducing the impact of dietary cholesterol. In addition, the fish oil diet fed to these hamsters is low in n-6 PUFA, contributing to a lower N6:N3 ratio. This indicates that while a higher n-3 fatty acid concentration should be beneficial, it is apparently not in F_1B hamsters, and a balanced N6:N3 ratio is necessary to lower plasma cholesterol concentrations. In addition, a lower

n-6 fatty acid concentration may affect the hamsters response to SFA. It should also be noted that the C14:0 to C16:0 ratio is much higher in the fish oil diet than the MUFA and PUFA diets. It is known that 14:0 can elevate plasma cholesterol concentrations, while 16:0 is generally considered to be a neutral fatty acid (Hayes, 1995). Therefore it is possible that the elevations in plasma lipid and lipoprotein cholesterol concentrations seen in F_1B on the fish oil diet may be partially due to a higher level of SFA (particularly 14:0) than the MUFA and N6:N3 diets.

4.1.2 Monounsaturated Fatty Acids and the Plasma Lipid Profile

The effects of a diet high in MUFA are mixed results in the scientific community. It was originally thought that supplementation of the diet with MUFA exerted neutral effects on the improvement of plasma lipids and lipoprotein profiles (Keys *et al.,* 1965; Hegsted *et al.,* 1965). It has been demonstrated however, that MUFA may possess cardioprotective properties that may equate those of PUFA (Gardner & Kraemer, 1995). Earlier studies in humans have shown favourable effects of MUFA on the plasma lipid profile when substituted for SFA (Mattson & Grundy, 1985; Katan *et al.* 1995). More recent studies, and certainly the evidence for the beneficial effects of the Mediterranean diet have provided a strong basis for supplementation of the diet with MUFA (Kris-Etherton *et al.,* 1999; Hiraoka-Yamamoto *et al.,* 2004). In our study both strains of hamsters on the MUFA diet had reduced total plasma cholesterol, TG, FC, and CE concentrations compared to those animals on the fish oil diet. This is consistent with the results of several other studies investigating the effects of MUFA supplementation on the lipid profile in hamsters (Sessions & Salter, 1994; Trautwein *et al.,* 1999). In hamsters

fed a mid- or high-oleic oil diet, significant reductions in plasma cholesterol were noted compared to a high-linoleic oil diet (Nicolosi *et al.,* 2002). Reduced plasma cholesterol concentrations were also reported in hamsters fed a diet rich in olive oil compared to those fed coconut oil (Mangiapane *et al.,* 1999). In addition, several authors have also noted a significant decrease in the development of atherosclerotic lesions in GS hamsters on a MUFA diet (Mangiapane *et al.,* 1999; Nicolosi *et al.,* 2002). There are studies however, which have also demonstrated that MUFA and PUFA exert similar effects on plasma lipid and lipoprotein profiles. GS hamsters on both hypercholesterolemic PUFA and MUFA diets show no significant differences between plasma TG concentrations after a two-week feeding period (Nicolosi *et al.,* 2002). It should be noted however, that the source of PUFA was not marine oil, but sunflower oil. This same study however, did demonstrate that GS hamsters on the MUFA diet developed atherosclerosis later compared to animals on the PUFA diet.

4.1.3 The N6:N3 Ratio and Plasma Lipid Composition

There is evidence that consumption of an optimal ratio of n-6 and n-3 fatty acids is valuable for maintaining a favourable lipid and lipoprotein profile (Jump & Clarke, 1999). It is estimated that the current n-6:n-3 ratio in Western populations is 15-20:1, due mainly to an increase in the consumption of foods such as vegetable oils, which are increasingly rich in n-6 fatty acids (Simopoulos, 2002). An elevated n-6:n-3 ratio is potentially harmful as high concentrations of n-6 fatty acids within the body are thought to promote inflammation and platelet aggregation (Simopoulous, 1999). On the other hand providing an n-6:n-3 ratio of 4:1 has been shown to improve cardiovascular health

in humans (de Lorgeril *et al.*, 1994). There are very few studies however, on the effects of diets with specific n-6:n-3 ratios in both humans and animals. Since we previously found that fish oil induced hyperlipidemia in F_1B hamsters, we examined whether an n-6:n-3 ratio of 5 had the ability to lower plasma lipoprotein concentrations as opposed to fish oil alone. This n-6:n-3 ratio proved to be beneficial for the plasma lipid profile in both F_1B and GS hamsters compared to hamsters fed the fish oil diet where the total plasma cholesterol, TG, FC, and CE concentrations were significantly reduced on the N6:N3 diet. Interestingly, we also noted a trend for an increase in plasma total cholesterol, TG and CE concentrations in F_1B hamsters on the N6:N3 diet compared to those on the MUFA diet. This suggests that the presence of even a small amount of fish oil in the diet has potentially damaging effects on the plasma lipid profile in hamsters. This change in lipid profile is most notable in the increase in LDL-cholesterol seen in F_1B hamsters on the fish oil diet and will de discussed in the next section.

4.1.4 Dietary Fats and VLDL Lipid Composition

Dietary fat has a significant effect on plasma VLDL-cholesterol, -TG, -FC, and -CE concentrations where F_1B and GS hamsters on the fish oil diet had highly elevated VLDL-lipid concentrations compared to those hamsters on the MUFA and N6:N3 diet. While these results are consistent with previous findings in our lab (De Silva *et al.*, 2003), fish oil is not typically known to induce hyperlipidemia. In fact, the anti-atherogenic properties associated with fish oil supplementation are due to its ability to decrease both plasma and VLDL-TG concentrations. Several studies conducted in hamsters however, have found that diets rich in fish oil and cholesterol increase VLDL-

cholesterol concentrations, but have no effect on VLDL-TG concentrations (Surette *et al.*, 1992; Lin *et al.*, 1995; Kubow *et al.*, 1996; Lu *et al.*, 1996). In this study, we found that VLDL-TG concentrations were elevated in F_1B and GS hamsters on the fish oil diet, suggesting that fish oil is decreasing the clearance of TG-rich lipoproteins.

It has been suggested that MUFA exert similar effects on the plasma lipoprotein profile as the n-3 PUFA (Kris-Etherton *et al.*, 1999). In this study however, VLDL-lipid concentrations were the lowest in F_1B and GS hamsters on the MUFA diet. These findings are consistent with Trautwein *et al.* (1999) who found that VLDL-TG concentrations, and the VLDL/HDL ratio were significantly decreased in hamsters on a MUFA diet. It should be noted however, that the beneficial effects of MUFA seen by these authors were compared to that of a diet rich in SFA. In contrast to our findings, when a diet rich in MUFA was compared to a diet rich in PUFA, MUFA had no effects on VLDL lipid concentrations in GS hamsters, whereas PUFA from sunflower oil reduced them (Sessions & Salter, 1994).

We also found that there is a trend for an increase in VLDL-FC and -CE concentrations in hamsters on the N6:N3 diet compared to hamsters on the MUFA diet. Omega-6 fatty acids typically reduce lipoprotein-cholesterol concentrations (Wijendran & Hayes, 2004), and have been shown to exert a greater plasma lipid lowering effect than MUFA (Kris-Etherton *et al.*, 2004). Therefore, the slight increase in VLDL lipid concentrations observed in hamsters fed the N6:N3 diet is likely due to the addition of fish oil, which has been shown in this study to drastically elevate plasma lipid and lipoprotein parameters.

The findings from this study on the effects of dietary fat on VLDL lipid concentrations are puzzling. Given that fish oils are known to possess hypotriglyceridemic properties and decrease VLDL synthesis and secretion in hamsters, it is likely that elevated VLDL-lipid concentrations are a consequence of inhibited hepatic uptake, and not the enhanced synthesis of VLDL.

4.1.5 Dietary Fats and LDL Lipid Composition

The composition of the LDL particle is considered to be a strong-predictor of CVD development. There is a reported 1.5% decrease in CVD incidence for every 1% reduction in LDL-cholesterol concentrations (Kris-Etherton *et al.*, 1999). In this study, there was a significant effect of diet on LDL-cholesterol, -TG, -FC, and -CE concentrations. Fish oil appeared to significantly increase these lipid parameters, whereas the MUFA and N6:N3 diets lowered LDL lipid concentrations. We also note a small increase in all LDL-lipid parameters in hamsters in response to the N6:N3 diet.

The controversy surrounding fish oil supplementation arises from its potentially harmful effects on LDL cholesterol concentrations. There is a growing body of evidence indicating that fish oil increases LDL-cholesterol concentrations in humans. Fish oil supplementation to hyperlipidemic and normolipidemic subjects has been shown to increase LDL-cholesterol concentrations (Sullivan *et al.*, 1986; Wilt *et al,*, 1989; Kestin *et al.*, 1990; Hsu *et al.*, 2000; Montori *et al.*, 2000; Nestel *et al.*, 2000; Dewailly *et al.*, 2001; Farmer *et al.*, 2001). In addition, these increases in LDL-cholesterol have also been observed in hamsters (Surette *et al.*, 1992; Lin *et al.*, 1995; Lu *et al.*, 1996). In diets enriched with sardine oil, for example, a 90% increase in LDL cholesterol concentration was found in GS hamsters (Surette *et al.*, 1992). Similarly, significant increases in LDL-

cholesterol concentrations were observed in hamsters given n-3 fatty acids in comparison to both n-6 fatty acids, and diets rich in soybean oil (Lu *et al.*, 1996; Lin *et al.*, 1995). The mechanism behind this increase is unknown, and certainly puzzling considering the inhibitory effect of fish oil on VLDL secretion, and subsequent LDL production. It is thought that fish oils may increase the hydrolysis of VLDL to LDL, or alter lipoprotein composition, which can affect binding affinity for LPL and the LDLr, which will be discussed in later sections.

In addition, fish oil feeding to F_1B and GS hamsters significantly increased the TG content of LDL. These findings are reminiscent of an ALP, where diet-induced hypertriglyceridemia results in the enrichment of LDL and HDL with TG, lipoprotein particles that normally carry cholesterol. Fish oil has typically been shown to reduce the development of this phenotype, thus a fish oil-induced increase in LDL-TG concentrations is not common. There are reports however, that fish oil increases TG concentrations in hamsters (Hayes *et al.*, 1992), although this was mainly observed in whole plasma as opposed to specific fractions. Researchers speculate that elevated lipid concentrations during fish oil supplementation may be attributed to elevated VLDL lipid concentrations (Lu *et al.*, 1996).

In contrast to the effects of fish oil on LDL-lipid concentrations, hamsters on the MUFA diet had the lowest LDL-lipid concentrations. MUFA are typically thought to exert neutral effects on LDL-lipid levels. It has been argued that the effects of MUFA on LDL-lipid concentrations may not necessarily reflect the anti-atherogenic properties of MUFA, but rather the removal of other dietary fatty acids that are considered to be atherogenic. In this study however, we found that F_1B and GS hamsters on the MUFA

diet had markedly lower LDL-cholesterol, -TG, -FC, and -CE concentrations than those hamsters on the fish oil diet. Similarly, studies in GS hamsters have also shown significant decreases in LDL-cholesterol concentrations on diets enriched with olive oil compared to coconut oil, and sunflower oil (Sessions & Salter, 1994; Mangiapane *et al.*, 1999). In contrast to these findings however, comparison of a MUFA diet to a PUFA diet revealed no significant differences observed between LDL-lipid levels, furthering the hypothesis that MUFA have no effect on the plasma lipid profile (Nicolosi *et al.*, 2000). Interestingly however, these authors found greater fatty streak formation in hamsters fed the PUFA diet compared to the MUFA diet.

One of the most intriguing findings of our study is the effect of the N6:N3 diet. This diet was shown to increase LDL lipid concentrations compared to the MUFA diet, particularly in F_1B hamsters. Omega-6 fatty acids, particularly linoleic acid, are considered to have the most potent LDL-cholesterol lowering effects, at both high and low dietary intakes (Wijendran & Hayes, 2004). Thus in this study, the N6:N3 diet would ideally lower LDL-lipid parameters. Our results however provide further evidence that lipoprotein metabolism is altered when fish oil is added in the diet, and may be over-powering the potentially positive effects of a balanced n-6:n-3 ratio.

4.1.6 Dietary Fat and HDL Lipid Composition

The composition and concentration of plasma-HDL is a strong predictor of the development of atherogenesis. An inverse relationship between the risk of CVD and HDL-cholesterol concentrations has been established (Kris-Etherton *et al.*, 1999). Similar to VLDL and LDL particle composition, dietary fat intake plays a key role in HDL lipid

composition. In this study, there was a significant effect of diet on HDL-TG, -FC, and -CE concentrations, but not on HDL-cholesterol concentrations.

Fish oil feeding markedly increased HDL-TG and -FC, while HDL-CE concentrations were decreased in hamsters on the fish oil diet. The enrichment of HDL particles with TG is parallel to that seen in LDL particles during hypertriglyceridemia. HDL particles become susceptible to hydrolysis by hepatic lipase and the resultant HDL particle is small and deprived of cholesterol, resulting in a decrease in HDL-cholesterol concentrations, and subsequent increase in CVD risk (Austin, 2000). The enrichment of HDL-particles with TG during fish oil supplementation has not previously been reported in hamsters as the effects of fish oil on HDL composition are usually restricted to HDL-cholesterol concentrations.

The significant decrease in HDL-CE concentrations in fish oil fed hamsters compared to those on the other two diets is also an interesting finding and has been previously reported in our lab (De Silva *et al.*, 2004). This decrease can be attributed to the fact that n-3 fatty acids have been shown to have less affinity for the hepatic LCAT enzyme (Parks *et al.*, 1997, 1998). Poor esterification of HDL-FC to HDL-CE by LCAT may account for the decline in HDL-CE concentrations.

Our findings show no significant effect of diet on HDL-cholesterol concentrations. Similar studies in GS hamsters have shown that HDL-cholesterol concentrations are unaffected by fish oil supplementation (Kubow *et al.*, 1996). There are other studies however, that demonstrate that fish oil significantly decreases HDL-cholesterol concentrations (Ward & Clarkson, 1985; Surette *et al.*, 1992; Lin *et al.*, 1995;

Lu *et al.*, 1996). In humans, there are also reports of both increases and neutral effects of fish oil on HDL-cholesterol concentrations (Harris, 1988, 1996).

The lowest HDL-TG and -FC concentrations in F_1B and GS hamsters in this study were found on the MUFA diet. HDL-CE concentrations however were elevated, and there was no effect on HDL-cholesterol concentrations in the F_1B or GS hamsters. In humans, dietary MUFA reportedly have no effect on HDL-cholesterol concentrations (Mattson & Grundy, 1985; Montoya *et al.*, 2002; Nicklas *et al.*, 2002). Similarly, HDL-cholesterol concentrations in hamsters fed diets rich in olive oil or safflower oil remain unaffected compared to hamsters fed diets rich in SFA (Kubow *et al.*, 1996; Mangiapane *et al.*, 1999). In contrast however, MUFA diets were shown to significantly decrease the risk of atherogenesis by decreasing the VLDL:HDL ratio in GS hamsters (Trautwein *et al*, 1999). While diet does not affect HDL-cholesterol concentrations in this study, we do see a decrease in HDL-TG concentrations on the MUFA diet, shifting from an ALP on the fish oil diet to a more positive lipoprotein profile. The effects of MUFA on HDL-TG concentrations have not previously been reported in hamsters. In humans, however, MUFA have been shown to possess hypotriglyceridemic properties (Mensink & Katan, 1992).

The N6:N3 diet appeared to exert similar effects as the MUFA diet on HDL-FC, and -CE concentrations, with a lack of influence on HDL-cholesterol concentrations. These findings are consistent with the literature where n-6 fatty acids typically have negligible effects on HDL-cholesterol concentrations. There was however, an increase in HDL-TG concentrations in hamsters on the N6:N3 diet compared to those on the MUFA diet. These findings are consistent with the effects of the N6:N3 diet on VLDL- and

LDL-TG concentrations. It is obvious then, that the lipoprotein metabolism of F_1B and GS hamsters is affected by the addition of fish oil in the N6:N3 diet.

4.1.7 Comparison of Plasma Lipids in F_1B and GS Hamsters

It has long been established that heterogeneity in genetic background contributes to the varied plasma lipid in response to dietary fat intake (Ahren *et al.*, 1957). Previously in our lab, we found that the F_1B hamster responded to a fish oil diet with severe dyslipidemia. In humans we also see a differential response to dietary fatty acid intake. Since the F_1B hamster is an inbred strain, we decided to compare this hamster to the outbred GS hamsters to determine if this unusual response to fish oil was strain-specific. Several investigators have reported that there are variations in plasma lipid concentrations between hamster strains (Kowala *et al.*, 1991). Therefore, we hypothesized that F_1B and GS hamsters would respond differently to the fish oil, MUFA, and N6:N3 diets. We found that fish oil induced hyperlipidemia is much more pronounced in F_1B than GS hamsters. There was a significant effect of diet and strain on plasma cholesterol, TG, FC, and CE concentrations, where F_1B hamsters on the fish oil diet had dramatically elevated concentrations compared to GS hamsters. In addition, F_1B hamsters on the fish oil diet had milky plasma packed with chylomicron-like particles which was not observed in fish oil fed GS hamsters.

The possibility of heterogeneity in genetic background between hamster strains is well established (Kowala *et al.*, 1991; Trautwein *et al.*, 1993). While both F_1B and GS hamsters have been shown to develop atherosclerosis on diets rich in fat and cholesterol, F_1B hamsters have been shown to be more susceptible to diet-induced atherosclerosis

than other hamster strains (Kowala *et al.*, 1991). Dorfman *et al.* (2003) for instance, found that F_1B hamsters have markedly higher total cholesterol concentrations than GS hamsters on diets rich in SFA. These authors also find significantly higher fasting plasma TG concentrations in F_1B compared to GS hamsters. Further comparison of the F_1B hamster to the DSNI hamster strain has also shown dissimilarities between strains where F_1B hamsters had significantly elevated total cholesterol and TG concentrations, as well as increases in TG-rich lipoprotein cholesterol concentrations compared to DSNI hamsters (McAteer *et al.*, 2003).

The FPLC lipoprotein profile from this study also indicates that different responses to a high fat fish oil diet occur between the two hamster strains. Fish oil-fed F_1B hamsters have a significant portion of their plasma cholesterol in the VLDL fraction compared to GS hamsters. We also find a significant effect of strain on VLDL, LDL, and HDL lipid concentrations. Differences in the lipoprotein profile are also reported in the literature where GS hamsters typically have increases in both LDL- and HDL-cholesterol in response to an atherogenic diet (Hayes *et al.*, 1992). F_1B hamsters on the other hand, have highly elevated LDL-cholesterol concentrations without appreciable effects on HDL-cholesterol, further suggesting genotypic differences between hamster strains (Kowala *et al.*, 1991).

Dietary supplementation of both MUFA and PUFA to humans has a variety of effects on plasma lipid concentrations with either increases (Wilt *et al.*, 1989; Kestin *et al.*, 1990; Nestel *et al.*, 2000; Dewailly *et al.*, 2001) or decreases (Harper & Jacobson, 2001). It is quite possible that differences within our genetic background contribute to this diverse response. The exact variation in genetic background responsible for the

reported response to dietary fat is unknown, but is likely attributable to numerous factors. The results from this study indicate a possible variation in the clearance of lipids from the plasma which include diversity in LPL activity.

4.1.8 Dietary Fats and Hepatic Lipid Concentrations

Dietary fat has a significant effect on hepatic total cholesterol TG, and CE concentrations, where fish oil feeding to both F_1B and GS hamsters significantly increased hepatic lipid concentrations. Similar results have been reported in our lab (de Silva *et al.*, 2004), and others (Gaiva *et al.*, 2003), where higher hepatic lipid concentrations have been observed in animals fed fish oil diets. The increase in hepatic cholesterol concentrations in fish oil fed hamsters also indicates an excess in cholesterol available for esterification, hence the increase in hepatic CE concentrations. Similarly, the decrease in hepatic cholesterol available for esterification in hamsters on the MUFA and N6:N3 diets accounts for the observed decrease in hepatic CE concentrations. These findings however oppose studies where MUFA have been shown to promote the storage of hepatic CE in hamsters (Sessions & Salter, 1994; Salter *et al.*, 1998), resulting in elevated hepatic CE concentrations.

The reason for the observed increase in hepatic lipid concentrations in fish oil fed hamsters cannot be explained. However, the development of hyperlipidemia and suspected decrease in lipoprotein clearance suggests that the increase in hepatic lipid content is due to enhanced hepatic lipogenesis rather than hepatic influx of dietary fat. Several hepatic enzymes such as ACAT, which is responsible for the hepatic

esterification of cholesterol, are known to be influenced by dietary fatty acid intake and availability and may be upregulated in these animals.

In addition to the dietary effects on hepatic lipids, the difference in hamster strain in response to dietary fat is also apparent in hepatic TG concentrations. GS hamsters on all three diets had a 2-fold increase in hepatic TG concentrations compared to F_1B hamsters, while hepatic cholesterol, FC, and CE concentrations remain unaffected by hamster strain. The observed decrease in hepatic TG concentrations in F_1B hamsters is expected due to the presence of milky plasma and highly elevated plasma lipid and lipoprotein TG concentrations. This suggests that despite elevated plasma and lipoprotein TG concentrations, the F_1B hamsters are neither storing, nor synthesizing an excess of hepatic TG. This further highlights the fact that elevated plasma cholesterol and TG concentrations are perhaps due to alterations in clearance of lipoproteins from the plasma and not the overproduction of hepatic-derived lipoproteins.

4.2 Triglyceride-Rich Lipoprotein Metabolism

4.2.1 Plasma Apolipoprotein B Protein Expression

Apolipoprotein B is a key apoprotein involved in lipoprotein metabolism. In addition to its structural role in chylomicron, VLDL, and LDL formation, apoB48 and apoB100 are required for the synthesis and secretion of chylomicrons and VLDL respectively. Due to the presence of milky plasma, and elevated plasma TG concentration, Western blot analysis was performed in this study using primary antibodies against human apoB to determine the origin of TG-rich lipoproteins. Both

apoB48 and apoB100 were detected, indicating the presence of both intestinally and hepatically derived lipoproteins in F_1B and GS hamsters after a 14-hour fast.

We are the first to observe that F_1B and GS hamsters on a fish oil diet had significantly higher plasma apoB48 and apoB100 protein expression than hamsters on the MUFA and N6:N3 diets. Typically, n-3 fatty acids found in fish oil are known to decrease the synthesis and secretion of apoB by increasing the intracellular rate of apoB degradation (Kendrick & Higgins, 1999). There are reports however, with similar findings to our study where increases in apoB concentrations are observed upon supplementation of fish oil in HepG2 cells (Arrol *et al.*, 2000).

In contrast to fish oil feeding, F_1B and GS hamsters on the MUFA diet had negligible amounts of apoB100, and the absence of apoB48 protein expression. These findings are consistent with both human and animal studies where apoB concentrations are reduced by MUFA (Wahrburg *et al.*, 1992; Zambon *et al.*, 1995; Aro *et al.*, 1998; Desroches *et al.*, 2004).

The supplementation of the N6:N3 diet produced very interesting changes in apoB100 protein expression. The polyunsaturated n-6 fatty acids are known to significantly increase apoB expression, which is another aspect that contributes to the danger of elevated consumption of these fatty acids and an unbalanced n-6:n-3 ratio. Thus the decrease in apoB synthesis typically induced by n-3 fatty acids is necessary to prevent the oversecretion of apoB and its associated lipoproteins. In this study however, F_1B hamsters on the N6:N3 diet had elevated apoB48 and apoB100 protein expression, signifying an over-powering combination of fish oil and n-6 fatty acids on apoB protein expression.

In addition to dietary effects on apoB protein expression, the effect of hamster strain is also apparent. While apoB48 and apoB100 protein expression were elevated in both F_1B and GS hamsters on the fish oil diet, this effect was more obvious in F_1B hamsters. Similarly, apoB48 and apoB100 protein expression was elevated in F_1B hamsters on the N6:N3 diet, but not in GS hamsters on the N6:N3 diet. The apoB concentrations in these hamsters parallel their differences in plasma lipid and lipoprotein concentrations as well, where F_1B hamsters had milky plasma, and significantly greater VLDL and LDL lipid concentrations than GS hamsters. We are the first to demonstrate variation in apoB protein expression between hamster strains in response to various dietary fats. These differences further highlight the heterogeneity between animal strains, and suggest differential regulation of lipoprotein metabolism by dietary fat in these animals.

There are two plausible explanations for the variation in apoB protein expression. These include either the enhanced synthesis and secretion of apoB and apoB-containing lipoproteins, or a decrease in the clearance of these lipoproteins from the plasma. The latter of these two hypotheses is the most conceivable. The amount of apoB secreted by the intestine and liver is in excess of the requirement for the synthesis of lipoproteins. ApoB is quickly degraded when unused. Therefore, the presence of apoB48 in the plasma after a 14-hour fast is suggestive not of enhanced apoB48 synthesis, but of decreased catabolism. Furthermore, while fish oils are known to increase apoB concentrations, it was subsequently shown that hepatic TG accumulation was the dominant influence on this apoB secretion in human hepatocytes (Arrol *et al.*, 2000). It was previously mentioned that fish oil feeding to F_1B hamsters resulted in significantly less hepatic TG

accumulation than in GS hamsters, thus the availability of lipid substrates is an unlikely factor for the elevation in apoB protein expression. While the clearance of apoB-containing lipoproteins appears to be the most likely explanation for the variation in apoB protein expression, the synthesis, secretion, and degradation of apoB were not measured in this study, therefore we cannot exclude the possibility that these aspects of apoB metabolism were influenced by dietary fat or animal strain.

4.2.2 Dietary Fats and MTTP Activity

The next step involved in the synthesis of TG-rich lipoproteins involves the transfer of lipid to the newly synthesized, nascent, apoB particle for the formation, and subsequent secretion of intestinally derived chylomicrons, or hepatic VLDL, which is catalyzed by MTTP. The activity of MTTP in this study was measured in both the intestine and the liver to determine if the substantial increase in chylomicron-like particles, VLDL, and apoB protein expression were due to an increase in their synthesis induced by fish oil. We hypothesized that fish oil feeding in F_1B hamsters would increase MTTP activity to a greater extent than the MUFA and N6:N3 diets. This would allow us to partially attribute the increase in TG-rich lipoproteins, and apoB expression to enhanced MTTP activity. We found no effects of dietary fat or hamster strain on MTTP activity.

The rate of TG-rich lipoprotein secretion is strongly affected by the rate of endogenous production, which is particularly influenced by the availability of intracellular TG and fatty acids. The availability of TG appears to be critical for the stimulatory effect of MTTP on chylomicrons and VLDL (Salter *et al.*, 1998). We have

found however, that F_1B hamsters on the fish oil diet in particular had significantly lower hepatic TG concentrations than GS hamsters. If we had found that dietary fat had an influence on MTTP activity, one would expect a decrease, rather than an increase in MTTP activity.

Fatty acid availability, particularly the quality of fatty acid, is also known to affect MTTP activity (Salter *et al.*, 1998). Studies on MTTP mRNA expression in hamster hepatocytes have shown that diets rich in SFA increase hepatic and intestinal MTTP mRNA expression (Lin *et al.*, 1994; Bennett *et al.*, 1995). Recently, it has also been shown that MTTP mRNA expression is unaffected by incubation with either n-3, or n-6 enriched chylomicron remnants (Botham *et al.*, 2003). These studies however, fail to mention if the effects on MTTP mRNA expression are correlated in any way with MTTP activity.

The lack of effect of dietary fat or animal strain on both hepatic and intestinal MTTP activity, in addition to the abundance of evidence suggesting that fish oils decrease VLDL-secretion, further indicates that it is not the secretion of TG-rich lipoproteins that is affected, but the clearance of these lipoproteins and their remnants from the plasma.

4.2.3 Dietary Fats and Lipoprotein Lipase Activity

The presence of apoB48 and apoB100 and highly elevated plasma and VLDL-cholesterol and -TG concentrations after a 14-hour fast in fish oil fed F_1B hamsters indicates an inhibition in the clearance of TG-rich lipoproteins. Lipoprotein lipase is the rate-limiting determinant for the hydrolysis of chylomicrons and VLDL within the

plasma, and plays a key role in TG-rich lipoprotein clearance. In this study, there was a significant effect of strain, but not diet on post-heparin LPL activity. We found that F_1B hamsters had significantly lower post-heparin LPL activity than GS hamsters indicating variations in genetic background between these two strains. This decrease in LPL activity in F_1B hamsters provides a partial explanation for the severe hyperlipidemia seen in fish oil-fed F_1B hamsters compared to GS hamsters. Similar results have been found in F_1B hamsters, where this strain had significantly lower post-heparin LPL activity after 6 months compared to DSNI hamsters (McAteer *et al.*, 2003). This response however, was dependent on dietary cholesterol intake.

In contrast to the effects of animal strain, post-heparin LPL activity was unaffected by the FO, MUFA, or N6:N3 diets used in this study. These findings are consistent with the majority of the literature in both humans and animal models which indicate that dietary fatty acids, specifically the n-3 fatty acids from fish oils, do not influence LPL activity, as LPL activity is mainly regulated by insulin concentrations (Park, 2003). It is in contrast however with studies by Kasim-Karis *et al.* (1995), and Khan *et al.* (2002) who demonstrate that fish oils substantially increase post-heparin LPL activity.

There are several possible explanations for both the results from this study, and the inconsistencies that exist in the literature. The measurement of LPL activity as determined by the post-heparin LPL assay is actually based on the amount of LPL released from the tissue by the injection of heparin. Thus, this endogenous LPL activity does not necessarily reflect *in vivo* LPL activity or capability of chylomicron and VLDL hydrolysis. In addition, the LPL enzyme is known to be saturated at plasma TG

concentrations above 200mg/dL. Therefore, in this study, where plasma TG concentrations reach levels upwards of 1900 mg/dL at the highest, and 350mg/dL at the lowest, the LPL enzyme is saturated and may not allow for reflection of the effects of dietary fatty acids. This may partially explain why we find only a trend for a decrease in LPL activity in fish oil-fed hamsters, as opposed to a significant outcome.

In addition to the level of LPL activity, dietary fatty acids are known to alter lipoprotein composition and the interaction of lipoproteins with LPL. While LPL activity may not be influenced by dietary fat, the amount and composition of lipoproteins may affect their affinity for LPL. Chylomicrons and VLDL for example, are known to compete for the LPL enzyme, where chylomicrons are preferentially hydrolysed (Griffin *et al.*, 2001; Karpe & Hultin, 1995; Berr *et al.*, 1992; Potts *et al.*, 1991; Brunzell *et al.*, 1979). Therefore, in this study, the abundance of TG-rich lipoproteins namely chylomicrons, as represented by the presence of apoB48 and apoB100, and VLDL as seen in the FPLC profile, might compete for LPL leading to a potential decrease in the hydrolysis of both chylomicrons and VLDL, and an increase in their circulating plasma concentrations.

It is also known that the composition of the lipoprotein particle affects LPL-mediated hydrolysis (Griffin, 2001). Lipoproteins which consist primarily of n-3 fatty acids EPA and DHA for example, are thought to be smaller, TG-poor, and resistant to LPL-mediated hydrolysis (Botham *et al.*, 1997; Levy & Herzberg, 1999; Oliveira *et al.*, 1997). Previous work in our lab has shown that F_1B hamsters on a fish oil diet have smaller VLDL particles compared to hamsters on a diet containing lard and safflower oil (de Silva, unpublished results). On the other hand however, it is thought that this

resistance is beneficial as there is less competition with chylomicrons for LPL and thus a reduction in postprandial lipemia (Griffin, 2001). The size of lipoprotein particles and their affinity for the LPL enzyme was beyond the scope of this study. It is quite possible, however, that changes in lipoprotein composition and size may affect their subsequent hydrolysis, regardless of LPL activity. This provides a possible explanation for the non-significant decrease in LPL activity in fish oil fed hamsters.

4.2.4 Dietary Fats and LDL Receptor mRNA Expression

The LDLr is key in the removal and clearance of lipoprotein cholesterol from the plasma. It has been reported that the LDLr can also mediate the removal of chylomicron remnants (Faria *et al.*, 1996). In this study, the effect of diet and strain on LDLr mRNA abundance was examined to determine if alterations in LDLr mRNA expression were partially responsible for elevated plasma lipid and lipoprotein concentrations. Dietary fats are known to regulate hepatic LDLr mRNA expression (Horton *et al.*, 1993, 1994). In addition, LDLr mRNA expression has been shown to be negatively correlated with LDL-cholesterol concentrations (Soutar *et al.*, 1986; Nanjee *et al.*, 1989; Wilkinson *et al.*, 1998).

Both n-6 and n-3 polyunsaturated fatty acids have been shown to suppress LDLr mRNA abundance (Lindsey *et al.*, 1992; Surette *et al.*, 1992; Wilkinson *et al.*, 1998). These findings provide a link between the increases in plasma LDL-cholesterol concentrations that occur with dietary fish oil supplementation. In contrast, MUFA have been shown to increase LDLr mRNA expression in humans and hamsters (Daumerie *et al.*, 1992; Kurushima *et al.*, 1995; Rumsey *et al.*, 1995; Gill *et al.*, 2003). In this study

however, there was no significant effect of either diet or hamster strain on LDLr mRNA expression.

The lack of influence of diet and animal strain on LDLr mRNA expression in this study does not exclude the possibility that LDLr activity or binding affinity are affected by these two endpoints. Dietary fatty acids have been shown to alter the LDLr protein, distribution rate, and recycling rate, none of which were examined in this study (Spady *et al.*, 1995). Alterations in any of these aspects by dietary fat or animal strain may account for the observed increases in plasma lipid and lipoprotein concentrations. In addition, the lipoprotein particle size is associated with the binding affinity of these particles to the LDLr (Nigon *et al.*, 1991). Previous work in our lab has shown that fish oil-fed F_1B hamsters have larger LDL particles than hamster on diets rich in lard and safflower oil (de Silva *et al.*, 2004). Studies in fish oil fed subjects have also reported increases in LDL particle size (Sanchez-Muniz *et al.*, 1999). In addition, it has been clearly shown that variation in lipoprotein particle size, especially very small or very large lipoprotein particles can decrease the affinity for the LDLr (Nigon *et al.*, 1991). Therefore, in this study, modification of the fatty acid composition of lipoprotein particles, or alterations in the size of lipoprotein particles by either dietary fat or hamster strain may affect the binding of these particles to the LDL receptor, resulting in the observed hyperlipidemia.

4.2.5 Dietary Fats and Apolipoprotein E Protein Expression

ApoE plays a critical role in the catabolism of the TG-rich chylomicrons and VLDL. The LDLr mediates TG-rich lipoprotein clearance as it has a high binding affinity for apoE (Faria *et al.*, 1996). Plasma apoE protein expression was measured in this study

to determine if dietary fat or hamster strain may regulate the expression of apoE and hence play a role in the catabolism of TG-rich lipoproteins. We hypothesized that fish oil feeding to F_1B hamsters would result in the down-regulation of apoE protein expression. This would provide a plausible case for the variation in the extent of hypertriglyceridemia, as well as for the development of hypertriglyceridemia due to a decrease in the clearance of TG-rich lipoproteins. Our hypothesis was primarily based upon studies conducted in apoE knockout mice, where fish oil feeding to these mice induced hypertriglyceridemia attributed to the absence of apoE (Asset *et al.*, 2001). These authors also determined that apoE was necessary for fish oil to actuate its hypotriglyceridemic properties. In this study however, our findings were the complete opposite of our hypothesis.

Fish oil-fed hamsters had elevated plasma apoE concentrations compared to those hamsters fed the MUFA and N6:N3 diets. The effects of specific fatty acids on plasma apoE concentrations are not extensively researched, and we are the first to investigate this phenomenon in hamsters. It has been shown however, that subjects fed a fish oil diet have significantly lower apoE concentrations compared to baseline (Buckley *et al.*, 2005). MUFA feeding to adult men has also been shown to decrease apoE concentrations compared to diets rich in SFA (Jackson *et al.*, 2005).

There are several possible explanations for the observed alterations in apoE protein expression. A significant decrease in apoE concentrations is known to reduce the clearance, while a small increase will enhance the clearance of TG-rich lipoproteins (Zhang *et al.*, 1992; Matsushima *et al.*, 1999). ApoE however is only considered to be an anti-atherogenic apoprotein to a certain extent. Overexpression of apoE at levels of more

than 30g/L leads to hypertriglyceridemia (Huang *et al.*, 1998). An increasing body of evidence indicates that while apoE is postulated to be required for the functioning of LPL (Havel *et al.*, 1984), that elevated apoE induces hypertriglyceridemia through the inhibition of LPL-mediated hydrolysis of lipoproteins (McConathy *et al.*, 1989; Gomez-Coronado *et al.*, 1993; Rensen *et al.*, 1996). Therefore, our findings suggest that the elevated protein expression of apoE may affect the binding of lipoproteins to LPL, thereby decreasing the hydrolysis of chylomicrons and VLDL and contributing to the elevated postprandial TG concentrations. Furthermore, TG-rich lipoprotein particles are known to compete for apoE for subsequent hepatic removal (Westphal *et al.*, 2000). In this case, the increased presence of VLDL and chylomicron-like particles will compete for apoE. In parallel to the competition between these lipoproteins for LPL, the result is a decrease in the uptake of VLDL, chylomicron remnants, or both.

In addition to the effect of dietary fat on plasma apoE protein expression, there is also an obvious difference in the response of F_1B and GS hamsters. Fish oil fed F_1B hamsters have greater apoE protein expression than fish oil fed GS hamsters, while the abundance of apoE protein expression in MUFA and N6:N3 fed F_1B and GS hamsters is reversed. In F1B hamsters, the relative expression of apoE reflects the plasma concentration of TG-rich lipoproteins where elevated apoE-associated lipoproteins such as VLDL were elevated in the N6:N3 diet compared to the MUFA diet. In GS hamsters however, there is greater expression of apoE in MUFA fed hamsters, which is not reflective of plasma lipoprotein concentrations. These findings have not previously been reported in either F_1B or GS hamsters. In humans however, the existence of various apoE phenotypes are known to influence both the response to dietary fatty acids, and the levels

of apoE protein expression. The possibility exists then, that this heterogeneity in apoE phenotype may present itself in the hamster, considering the presence of both inbred and outbred strains. ApoE isoforms have not currently been reported in any animal models. Should the existence of apoE isoforms in hamsters be discovered, it would provide a novel and unique animal model for the investigation of gene-nutrient interactions.

4.3 Future Directions

It has previously been reported that the female parent strain to the F_1B hamster has a defect in the regulation of cholesterol metabolism, where cholesterol biosynthesis is not under feedback inhibition (Schaffner *et al.*, 1981). Thus, investigation into the parent strains of both F_1B and GS hamsters will allow the potential location of genotypic differences that may influence strain-specific responses to dietary fat. The quantification of apoE in this study would allow for the determination of plasma apoE concentration and also indicate if there was an actual interference with LPL activity. ELISA kits to determine apoE concentration are currently available, but only for the detection of human apoE. The development of an ELISA specific to hamster or rodent apoE would allow appropriate quantification and a solid answer to our hypothesis concerning the overexpression of apoE.

Further investigation into the role of specific apolipoproteins is also necessary, particularly those of the apoC family. ApoCII and apolipoprotein CIII (apoCIII) are key players in lipoprotein metabolism as they interact with both LPL and apoE to mediate the clearance of TG-rich lipoproteins. ApoCII is a ligand necessary for the activation of LPL, while apoCIII is known to be an inhibitor of the enzyme. Therefore, a decrease in apoCII

or increase in apoCIII concentration or production may account for the differences in LPL activity between animal strains. In addition, apoCIII has been shown to interfere with the binding of apoE containing lipoproteins to the LDLr due to the displacement of apoE. In our study then, elevated plasma apoE concentrations do not necessarily suggest that apoE is bound to TG-rich lipoproteins. Western blot analysis was conducted using antibodies against human apoCII and apoCIII, however we did not obtain any results as these antibodies were not specific for hamsters. Measurement of apoCII and apoCIII concentrations and rates of production would provide further insight into the dysregulation of lipoprotein metabolism in these hamsters.

4.4 Conclusions

The use of three potentially cardioprotective diets yielded a large variation in several aspects of lipoprotein metabolism. Fish oil feeding induced severe dyslipidemia, accumulation of apoB48- and apoB100-containing lipoprotein particles after a 14-hour fast, and highly elevated plasma apoB and apoE protein concentrations. In contrast, hamsters on a diet rich in MUFA had the lowest plasma lipid and lipoprotein concentrations, and distinctly lower apoB and apoE protein concentrations. Furthermore, a diet containing a beneficial n-6:n-3 ratio significantly lowered plasma lipid concentrations and apoprotein expression, however, trends for an increase in plasma lipid concentrations in comparison to the MUFA diet indicate that addition of fish oil in the diet has an adverse effect on lipoprotein metabolism in F_1B and GS hamsters. It is quite possible that there are additional components of fish oil that may affect the regulation of

lipids and lipoproteins in hamsters, however this hypothesis was beyond the scope of this thesis.

Absence of any dietary effect on MTTP activity and elevated apoB48 protein expression further suggest that the observed dyslipidemia induced by fish oil is a result of the hindrance of TG-rich lipoprotein clearance as opposed to the synthesis and secretion of these lipoproteins.

Comparison of the inbred F_1B hamster to the outbred, normal GS hamsters, allowed us to partially attribute the fish oil induced hyperlipidemia to diversity in animal strain. Decreased LPL concentrations and elevated apoB and apoE protein expression in F_1B hamsters allude to heterogeneity within genetic background that contributes to the varied response of these hamsters to dietary fat. Further investigation into lipoprotein metabolism in these strains will lead to a more detailed understanding of the link between phenotype and dietary fats. Further identification of polymorphisms within each animal strain in response to various unsaturated fats may also provide insight into the diverse and controversial effects of fish oil on cholesterol and lipoprotein metabolism in humans.

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