THE ROLE OF INCREASED CHOLESTERYL ESTER TRANSFER PROTEIN ACTIVITY IN TYPE 1 DIABETES AND IN CHOLESTEROLEMIC EFFECTS OF DIETARY FATS IN TRANSGENIC MICE

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ABSTRACT

In the first study of this two-phase dissertation, we investigated the relationships between CETP activity, plasma lipid profile, fatty acid composition of lipoproteins, and fasting plasma glucose in thirty-five type 1 diabetic children. CETP activity was positively correlated with fasting plasma glucose, CETP concentration, lecithin:cholesterol acyltransferase (LCAT) activity, total cholesterol, free cholesterol, LDL-C, and LDL-cholesteryl ester. LCAT activity was positively correlated with CETP activity, total cholesterol, free cholesterol, LDL-C, CETP concentration, and LDL-cholesteryl ester, while negatively correlated with cholesteryl ester to free cholesterol ratio. Subjects with high fasting plasma glucose levels (>6.39 mmol/l) had higher CETP and LCAT activities than subjects with normal glucose concentrations. The simultaneously increased CETP and LCAT activities may result in the accumulation of LDL-C in these subjects. After adjusting for plasma CETP concentration, measured by a sandwich ELISA developed in this study, CETP activity was positively correlated with C18:2ω6 in HDL_3-triglyceride, and C16:0 and C20:4ω6 in LDL-cholesteryl ester. The accumulation of C20:4ω6 LDL-cholesteryl ester may affect eicosanoid biosynthesis, and consequently result in higher risk of cardiovascular disease in type 1 diabetic patients.

In the second part of this dissertation, we examined the cholesterolemic effects of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids in

CETP transgenic and control mice. Transgenic mice showed similar cholesterolemic responses to dietary fats as humans. The SFA group had significantly higher plasma TC than the PUFA group, and higher LDL+VLDL-C than the MUFA group. In addition, the PUFA group had lower HDL-C than the MUFA group. Transgenic mice consuming diets high in MUFA and PUFA had lower TC than controls in the same diet groups. In the MUFA group, Transgenic mice had decreased LDL+VLDL-C concentration, a lower LDL+VLDL-C to TC ratio, and a higher HDL-C to TC ratio than controls.

In this dissertation, we showed that CETP may be a potential target for pharmacological treatment in type 1 diabetic patients to reduce the risk of atherosclerosis. Furthermore, diets high in MUFA may be a possible dietary modification to lower the incidence of cardiovascular disease in these patients with high CETP activity.

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PUBLICATIONS

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FIELDS OF STUDY

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CHAPTER 1

INTRODUCTION

1.1 Background

Cholesteryl ester transfer protein (CETP), a key enzyme in reverse cholesterol transport process and remodeling of lipoproteins, mediates the exchange between cholesteryl ester in HDL and triglyceride in apo B-containing lipoproteins. Through this process, extrahepatic cholesterol is redistributed to other tissues for utilization or to the liver for excretion. Studies with CETP transgenic mice and genetically CETP deficient humans indicated that CETP lowers HDL-cholesterol, which is known to have protective effects against atherosclerosis. However, the role of CETP in atherogenesis is still not clear. On the one hand, CETP activity is elevated in type 1 (Bagdade et al. 1991a) and type 2 (Bagdade et al. 1993) diabetic, hypertriglyceridemic (Bagdde et al. 1991b), and obese (Arai et al. 1994) subjects. This increased cholesteryl ester transfer has been suggested to be partially responsible for the high risk of cardiovascular disease in these populations. Transgenic mice expressing CETP also had higher incidence of atherosclerosis than wild type mice that do not carry this gene. On the other hand, the large and cholesteryl-ester rich HDL from CETP-deficient patients had less protective effect in preventing cholesterol accumulation in macrophages (Ishigami et al. 1994) and

lower ability in promoting cholesterol-efflux, compared to the lipoprotein from normal subjects (Ishigami et al. 1994, Ohta et al. 1995). In addition, transgenic mice co-expressing CETP and apo CIII genes had less aortic lesions than mice expression apo CIII alone (Hayek et al. 1995).

Many studies have examined the effects of different dietary fats on plasma lipid profiles in humans (see McNamara 1987 and Katan et al. 1994 for reviews). In general, saturated fatty acids, except stearic acid, when replacing carbohydrate in the diet, raise plasma total and LDL-cholesterol. On the other hand, monounsaturated and polyunsaturated fatty acids decrease total and LDL-cholesterol. The levels of elevated total (Expert Panel, 1988) and LDL-cholesterol (Frick et al. 1987) have both been linked to the increasing incidence of cardiovascular disease.

The mechanisms regulating the cholesterolemic effects of dietary fats are complicated and may include interactions among: 1) composition of lipoprotein surface or core (Shepherd et al. 1980), 2) hepatic LDL receptor activity (Daumerie et al. 1992), 3) hepatic VLDL production rate (Dietschy 1998), 4) apolipoprotein metabolism (Shepherd et al. 1978), 5) fecal sterol excretion (Grundy and Ahrens Jr. 1970), and 6) change in the activities of enzymes involved in lipid metabolism (Groener et al. 1991, Dietchy 1998).

1.2 Objectives

The purpose of this two-phase study, composed of a human and an animal experiment, was to investigate (1) the physiological role of increased CETP activity in

atherogenesis in type 1 diabetic children, (2) the biological factors responsible for the increased CETP activity in type 1 diabetic children, (3) the role of CETP in cholesterolemic effects of dietary fats in CETP transgenic mice, and (4) the possible dietary intervention to reduce the risk of atherosclerosis in subjects with increased CETP activity. The objectives of this study are described below:

1. To develop a cost- and time-effective sandwich enzyme-linked immunosorbent assay for plasma CETP concentration;
2. To investigate the relationship among CETP and lecithin:cholesterol acyl transferase (LCAT) activities, and plasma lipid profiles in type 1 diabetic children;
3. To investigate the relationship between CETP activity and plasma CETP concentration in type 1 diabetic children;
4. To identify the effect of glycemic control on CETP and LCAT activities;
5. To examine the specificity of CETP on various acyl groups of cholesteryl ester in lipoproteins;
6. To investigate the role of CETP in cholesterolemic response to various dietary fats in CETP transgenic mice;
7. To compare the difference in plasma lipid profile change between CETP transgenic and control mice consuming various dietary fats.

1.3 Hypotheses

The following null hypotheses tested in this study are based on a significant level of p value less than 0.05.

1. There is no correlation among CETP and LCAT activities, and plasma lipid profile in type 1 diabetic children.
2. There is no correlation between CETP activity and plasma CETP concentration in type 1 diabetic children.
3. There is no difference in CETP and LCAT activities in type 1 diabetic children with normal and high fasting plasma glucose levels.
4. CETP showed no specificity for any of the acyl groups of cholesteryl ester in lipoproteins.
5. There is no difference in plasma total cholesterol, HDL-cholesterol, LDL+VLDL-cholesterol, triglyceride, and CETP concentrations among CETP transgenic mice consuming AIN-93 diet, a low-fat diet, and diets rich in saturated, monounsaturated, and polyunsaturated fatty acids.
6. There is no difference in plasma total cholesterol, HDL-cholesterol, LDL+VLDL-cholesterol, and triglyceride between CETP transgenic and control mice consuming the same diet.

1.4 Significance of Study

Although it has been documented that CETP activity is increased in type 1 diabetic patients, the physiological effect of this accelerated cholesteryl ester transfer in atherogenesis is still not clear. The mechanism that mediates this increased activity is also mostly elusive. The results of this study could enhance our understanding in these aspects, and could indicate the possible targets for pharmacological interventions in these patients. Furthermore, the knowledge obtained from this study on the role of CETP in plasma lipid profile after consuming diets high in various fats could be used for dietary modification in order to lower the risk of cardiovascular disease in subjects with elevated CETP activity, such as type 1 diabetic patients.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Biological Functions, Structure, and Mechanisms of CETP

2.1.1 Overview

Cholesteryl ester transfer protein (CETP) mediates the exchange of cholesteryl ester in HDL and triglyceride in apo B-containing lipoproteins. It plays a crucial role in reverse cholesterol transport, and subsequently, cholesterol metabolism in the whole living system. Cholesteryl ester transfer activity is found in human, rat, guinea-pig, chicken, pig, dog, and rabbit, but not in the mouse (Ha and Barter, 1982).

Extensive study has been done on transgenic mice expressing the CETP gene, and on human subjects with genetic CETP deficiency. However, its role in atherogenesis is still debated and may depend on other biological and metabolic factors.

2.1.2 Studies of Transgenic Mice

Transgenic mice expressing the human CETP gene were first developed by Agellon et al. (Agellon et al. 1991). A 6.3-kilobase synthetic human CETP gene was linked to the mouse metallothionein-I promoter. The lipoprotein profile did not differ significantly

between transgenic and wild type mice, despite the detectable expression of CETP in transgenic animals. However, after the overexpression of CETP gene to about 2.5-fold of that in human plasma by dietary supplementation of Zn, CETP transgenic mice showed significantly lower HDL-C than wild type ones. Total cholesterol (TC) and LDL+VLDL-C did not differ (Agellon et al. 1991). In another strain of transgenic mice expressing simian CETP at the level up to 12-fold of that in humans, CETP activity was negatively correlated with TC, apo A-I, and apo A-I to apo B ratio. This strain of transgenic mice also had smaller HDL particle size, and higher TC and apo B concentration than wildtype animals after the challenge with a high-fat-high-cholesterol diet (Marotti et al. 1992).

In further studies with the human CETP transgenic mice, with the larger sample size, the researchers were able to demonstrate a statistically significant 20% decrease in HDL-C in CETP transgenic mice even without Zn supplementation (Hayek et al. 1992). There were still no differences in TC and LDL+VLDL-C between these two genotypes. However, the increase of LDL-C and VLDL-C became statistically significant in transgenic mice expressing even higher levels of human CETP (Jiang et al. 1993) and simian CETP (Marotti et al. 1993). The human CETP transgenic mice also had smaller HDL particle size, lower plasma apo A-I and TC concentration resulting from decreased HDL (Dinchuk et al. 1995).

When co-expressed with human apo A-I, the HDL-lowering effect of CETP became even more profound. Compared to wild type animals, this strain of double-transgenic mice had 35% and 66% reduction of HDL-C with and without Zn induction, respectively (Hayek et al. 1992). This enhanced effect may be due to the stronger interaction between CETP and HDL containing human apo A-I.

The increased CETP activity also down-regulated mRNA levels of hepatic LDL receptors, 3-hydroxy-3-methylglutaryl-coenzyme A reductase and 7-alpha-hydroxylase (Jiang et al. 1993). There were also increased hepatic TC, free cholesterol (FC), and cholesteryl ester (CE) concentrations in these animals (Jiang et al. 1993). The development of fatty liver by accumulation of TG has also been shown in simian-CETP transgenic mice (Blake et al. 1994).

The coexpression of human CETP and apo CIII in mice reduced HDL particle diameter to 8.8 nm from 10 nm in CIII transgenic and wild type mice (Hayek et al. 1993). The insertion of CETP into CIII transgenic mice, a hypertriglyceridemic model, further reduced HDL-C essentially by increasing HDL-CE fractional catabolic rate. This result suggested the critical role of CETP in the association of high TG with low HDL-C shown in humans. The CETP-apo CIII transgenic mice also had less incidence of proximal aorta lesions, as well as decreased lesion numbers and mean lesion area, than apo CIII mice when fed a high-fat-high-cholesterol diet, despite the lower HDL-C in the former (Hayek et al. 1995). This result contradicted the finding that transgenic mice expressing simian CETP gene alone had higher mean lesion area than wildtype animals (Marotti et al. 1993). Therefore, the role of CETP in atherogenesis may depend on the metabolic conditions.

The expression of CETP in transgenic mice produced HDL which is more efficient in promoting cholesterol efflux from rat hepatoma cell Fu5AH (Atger et al. 1995). However, the total cholesterol efflux potential of the transgenic serum measured by prolonged incubation of diluted serum with Fe5AH cells was lower than that with cells in wild type mice, which may result from the decreased HDL (Atger et al. 1995).

Furthermore, the plasma from transgenic mice coexpressing human CETP and apo A-I genes caused higher cholesterol efflux from fibroblasts, measured by incubation of undiluted plasma for several minutes, than from mice expressing apo A-I only (Francone et al. 1996). The inconsistency in the cholesterol efflux ability may be due to the different assay conditions. In the former study, the ability may largely depend on the larger HDL particles which have higher capacity for cholesterol. While in the latter study, the CETP-apo A-I mice showed higher levels of prebeta-HDL, which has been shown to be the main lipoprotein involved in the initiation step of cholesterol efflux from cells (Castro and Fielding 1988). However, one study showed no difference in cholesterol flux from the extrahepatic organs to the liver between simian-CETP transgenic and wild type mice (Osono et al. 1996).

In summary, the introduction of CETP into mice that usually do not carry this gene, markedly reduces HDL levels. It also facilitates the accumulation of cholesterol in the liver, and subsequently inhibits the expressions of proteins involved in hepatic cholesterol homeostasis. The physiological effect of CETP gene is still not clear, as studies on transgenic mice showed mixed results in the susceptiblity to atherosclerosis and the ability of HDL to promote cellular cholesterol efflux.

2.1.3 Studies in Rabbits

Inhibiting CETP activity in rabbits by injecting monoclonal anti-CETP resulted in an increase in HDL-CE and decrease in HDL-TG and VLDL CE/TG ratio, which implied

that CETP facilitates the exchange of HDL-CE and VLDL-TG (Whitlock et al. 1989). The clearance of HDL-C was also delayed after the inhibition (Whitlock et al. 1989).

Rabbits fed a high-fat-high-cholesterol diet showed higher plasma CETP activity and concentration, as well as plasma TC (Quig and Zilversmit 1988). The increase in CETP was later found to result from stimulation of CETP expression in the liver (Quinet et al. 1990). These results suggested the important roles of CETP in plasma lipid profile and HDL metabolism in rabbits.

In rabbits (Quinet et al. 1990) and primates (Quinet et al. 1991, Martin et al. 1993), a high-fat-high-cholesterol diet increased plasma CETP concentrations primarily by increasing CETP gene expression in the liver (Quinet et al. 1991, Jiang et al. 1992). This induction appeared to be independent of the LDL receptor pathway as it was still present in LDL receptor or apo E knockout mice (Masucci-Magoulas et al. 1996).

2.1.4 Human CETP Deficiency

The genetic basis of human CETP deficiency was first discovered in a Japanese family, where a point mutation converting G to A in the intron 14 (position +1) prevented normal splicing and resulted in the absence of CETP in the plasma (Brown et al. 1989). Further studies identified several other forms of mutations in various populations, which are listed in Table 2.1 (Hill and McQueen 1997). Further study in subjects with such an intron 14 G-A mutation revealed the marked increase in HDL_2, suggesting that the lipoprotein may be the primary donor of CE in the cholesterol transfer process (Inazu et al. 1990). The TG-rich HDL_2 after lipid transfer was converted into smaller HDL_3 by hepatic lipase. The increased HDL_2/HDL_3 observed in these subjects has been suggested

to be anti-atherogenic (Johansson et al. 1991). Another study on CETP deficient subjects demonstrated the lower CE and CE/TG ratio in IDL (Koizumi et al. 1991), which has also been related to lower risk of atherosclerosis (Tatami et al. 1981, Krauss 1987).

The CE-rich, TG-poor HDL_2 from CETP deficient subjects was significantly larger in size, while HDL_3 particles are normal in size and lipid composition, compared to normal people (Ishigami et al. 1994). The abnormal HDL_2 failed to prevent both the accumulation of CE in macrophages as induced by acetylated LDL and the removal of CE from cholesterol-loaded macrophages (Ishigami et al. 1994). HDL_3 from the patients appeared to be normal in both functions. HDL containing either apo A-I alone or both apo A-I and apo A-II from CETP deficient subjects also were CE-rich and had lower ability to prevent cholesterol accumulation in macrophages (Ohta et al. 1995). Ikewaki et al. (1993) demonstrated that CETP deficient subjects had a lower fractional catabolic rate of HDL_2 than normal people. Therefore, CETP may play a role in making HDL more protective against atherosclerosis by preventing CE accumulation in HDL.

In hyperalphalipoproteinemic subjects resulting from CETP deficiency, apo E-rich HDL, similar to HDLc found in animals fed a high cholesterol diet, accounted for 6-10% of the total HDL (Yamashita et al. 1990). This abnormal HDL contained 82% apo E and 18% apo A-I, had a density of 1.019-1.063 g/ml, but showed alpha-migration ability in agarose gel electrophoresis. Moreover, the apo E-rich HDL compared to LDL had higher affinity for, and lower number of particles required in saturation of, the LDL receptor on fibroblasts. The increase in apo E may result from the accumulation of CE in HDL and subsequently increased HDL core volume due to the lack of lipid transfer activity (Gordon et al. 1983).

LDL from two CETP deficient subjects was composed of two species with different size (Sakai et al. 1991), whereas LDL is homogeneous in normal humans. The size of the larger LDL particle found in CETP deficient subjects was identical to that in normal humans, while the other was considerably smaller. Small and dense LDL particles have been considered as a risk factor for cardiovascular disease (Crouse et al. 1985. Austin et al. 1988). Thus, CETP deficiency may also cause a proatherogenic lipid profile. The CE fatty acid composition in LDL in CETP deficient people was similar to that in its precursor, VLDL, indicating that CE in LDL was originally contained in VLDL, not obtained from cholesterol transfer. On the contrary, CE fatty acid composition in LDL from normal subjects resembled more closely to that in HDL, suggesting the result of CETP reaction (Bisgaier et al. 1991).

In a Japanese group of 201 hyperalphalipoproteinemic subjects, 67% were found to have mutations in intron 14 or exon 15 (D442: G) (Hirano et al. 1995). These two mutations are common even in the general Japanese population, with the estimated heterozygotic prevalence of 2% and 7%, respectively (Inazu et al. 1994). Although high HDL has been suggested to have a protective effect against CVD, 12 of these subjects were found to have atherosclerotic disease. In male CETP deficient subjects, hepatic lipase activity was significantly lower in patients with CVD than in those without (Hirano et al. 1995). HDL in CETP deficient subjects were larger and failed to prevent cellular cholesterol accumulation induced by acetylated LDL (Ishigami et al. 1994). Hepatic lipase is responsible for converting larger HDL_2 into smaller HDL_3, thereby reducing HDL_2 concentration (Kuusi et al. 1989, Blades et al. 1993). Therefore, combined with low hepatic lipase activity, CETP deficiency may further increase the

number of larger HDL particles which were less protective against cholesterol accumulation and had lower capability in delivering CE and apolipoproteins to hepatocytes (Collet et al. 1988). As a result, the effect of CETP deficiency, as well as hyperalphalipoproteinemia, on atherogenesis may depend on other metabolic parameters, such as hepatic lipase. However, it is worth pointing out that all of the CETP deficient men with CVD involved in this study had other risk factors, including smoking and hypertension.

In the Honolulu Heart Program study of males with pure Japanese ancestry living on the island of Oahu, 193 out of 3469 subjects (5.6%) were found to have a type of CETP mutation (Zhong et al. 1996). Compared to normal subjects in this study, the CETP deficient men had higher CHD despite their significantly higher HDL. The odds ratio were 1.43 (95% CI: 1.00-2.03, p=0.049) without any adjustment, and 1.68 (95% CI: 1.15-2.47, p=0.008) after adjusting for HDL levels. The higher prevalence of CHD in the CETP deficient men was especially significant in subjects with HDL values between 41 and 60 mg/dL, while there was no significant difference between these two genotypes with higher or lower HDL levels. Thus, the increased amount of HDL (higher than 60 mg/dL) may be able to compensate for the lower ability of abnormal HDL to prevent CHD.

To sum up, CETP deficiency in humans produces an abnormal lipid profile, including elevated HDL levels with lower protective effect against CVD, and small and dense LDL particles. The epidemiologic studies of CETP deficient subjects had mixed results on the incidence of CVD. The results suggested that CETP deficiency alone may not be a significant determinant of atherogenesis, while the interaction between CETP

and other physiological variables may play a more important role in the development of CVD.

2.1.5 Other Human Studies

In a study involving 50 normolipemic subjects, plasma CETP concentration was found to be 1.73±0.25 mg/L (mean±SD) ranging between 1.04 and 3.55. After gradient ultracentrifugation, the majority of CETP was associated with HDL_3 and very high density lipoprotein (VHDL) with only little amounts found in the free form. Most CETP-associated lipoprotein was found to migrate in the $alpha_2$ (pre-beta) position in agarose gel electrophoresis (Marcel et al. 1990).

VLDL remnants increased CETP activity in vitro because they were better substrates for CETP than the lipoprotein before lipolysis (Tall et al. 1984). The post-prandially accelerated lipoprotein lipase reaction (Sammett and Tall 1985) also increased CETP activity in vivo (Tall et al. 1986). In post-alimentary plasma, more CETP was bound to HDL and apoB-containing lipoproteins, while less CETP was present in the free form as determined by agarose chromatography (Tall et al. 1986).

Increased CETP activity was also discovered in dysbetalipoproteinemia, the accumulation of cholesteryl ester-rich VLDL remnants (beta-VLDL) in plasma (Tall et al. 1987). The accelerated CE transfer was considered to be the primary factor causing this atherogenic lipid profile.

Plasma CETP concentration was also elevated in patients with combined hyperlipidemia, hypercholesterolemia, and fasting chylomicronemia, but not hypertriglyceridemia (McPherson et al. 1991). These results suggested that high CETP

concentration is associated with the conditions where cholesterol delivery to extrahepatic tissue via LDL, beta-VLDL, or chylomicron remnants are increased. The correction of the abnormal lipid profile in chylomicronemic and dysbetalipoproteinemic patients by dietary modification normalized plasma CETP concentration, indicating that the increased CETP concentration may be secondary to atherogenic lipoprotein concentration. Increased plasma CETP concentration and activity may also be responsible for the atherogenic lipid profile in patients with nephrotic syndrome (Moulin et al. 1992) and glomerular proteinuria (Dullaart et al. 1993). In both cases increased apoB-containing lipoprotein and reduced HDL were found.

CETP activity is higher in obese subjects than in people with normal body weight (Arai et al. 1994). This effect may explain in part the low HDL levels constantly observed in obese people (Bertiere et al. 1988). In this group of obese subjects, CETP activity was positively correlated with body mass index, body fat ratio, and subcutaneous fat area, while negatively correlated with visceral fat/subcutaneous fat ratio. After reduction of body weight by 2 months of dietary therapy, both CETP activity and plasma concentration were decreased.

CETP activity was also higher in hypercholesterolemic (high LDL and normal TG) and combined hyperlipidemic (high LDL and TG) patients than in normolipidemic subjects (Tato et al. 1995). In combined hyperlipidemia, CETP activity was correlated positively with LDL-C and negatively with HDL-C, while only the positive correlation with LDL-C was significant in hypercholesterolemia.

Exercise training, which has been demonstrated to raise HDL-C and reduce LDL-C, could also reduce plasma CETP concentration (Seip et al. 1993). After 9 to 12 months of

endurance training, 57 elderly people reduced their plasma CETP concentration regardless of the weight change. The pretraining CETP concentration significantly associated with exercise-induced changes in HDL-C (r=-0.27) and LDL-C/HDL-C ratio (r=0.4). In other words, the lower the pretraining CETP concentration, the larger the HDL increase associated with exercise. The exercise-induced reduction in CETP concentration and LPL activity, combined with increased hepatic lipase activity, may be responsible for the lipid profile changes after training in this particular study (Seip et a. 1993). A preliminary study involving 5 highly trained runners showed that acute exercise could enhance the ability of HDL to promote cholesterol efflux from macrophages (Campaigne et al. 1993). However, the role of CETP in this effect could not be determined by this study because neither its concentration nor its activity was measured.

In summary, elevated CETP activity has been found in various populations at high risk of CVD. On the other hand, exercise training, which can reduce the risk of heart disease, seemed to reduce CETP activity. Consequently, CETP has been suggested to play a role in atherogenesis.

2.1.6 CETP and Type I Diabetes

An atherogenic lipid profile has been documented in insulin-dependent diabetes mellitus (IDDM) subjects with micro- and macrovascular complications, including elevated TC, LDL-C, and VLDL-C, and decreased HDL-C (Vannini et al. 1984, Laakso et al. 1986, Jensen et al. 1988, Dornan et al. 1982). CETP may play an important role in the development of abnormal lipid profiles in IDDM subjects because of its role in lipoprotein remodeling. In 1989, elevated CETP activity was first discovered in IDDM

subjects with micro- and macrovascular complications compared to IDDM subjects without complications and normal control subjects (Dullaart et al. 1989). Later, increased CE transfer was also found in normolipidemic IDDM subjects, compared to controls (Bagdade et al. 1991). In an effort to determine which lipoprotein is responsible for the increased CE transfer in IDDM, Bagdade et al. (1991) measured CETP activity under the conditions where one substrate lipoprotein was from IDDM subjects while the other one was from controls. It was revealed that only diabetic VLDL produced increased CE transfer similar to that observed in IDDM (Bagdade et al. 1991). Glycemic control also regulated CETP activity as fasting glucose and fructosamine positively correlated with CE mass transfer in IDDM (Ritter and Bagdade 1994).

In further study by the same group, IDDM VLDL and HDL_3 had a significantly higher ability to promote CE transfer, while HDL_2 and LDL from both control and IDDM showed similar effects. These results suggested that enhanced CE transfer in IDDM resulted from the accelerated CE-TG exchange between HDL_3 and VLDL (Ritter and Bagdade 1996).

CETP gene TaqIB polymorphism in IDDM subjects regulated lipid profile changes in response to a linoleic acid-enriched, low-cholesterol diet (Dullaart et al. 1997). In genotype B1B1, LDL+VLDL-C decreased and HDL-C increased after subjects followed the diet for one year, while HDL was lower and LDL+VLDL-C was unchanged in genotype B1B2. The lack of a difference in CETP concentrations between these two genotypes suggested that these two forms of CETP may vary in substrate specificities. Furthermore, a cross-sectional study revealed a 0.023 mmol/l increase in HDL-C in the presence of each B2 allele in IDDM subjects (Dullaart et al. 1998).

To sum up, accelerated CE transfer in IDDM patients is partially responsible for the abnormal lipid profile in this group of subjects. This increased CETP activity may also be related to various micro- and macro-vascular complications in these patients.

2.1.7 CETP and Type II Diabetes

CETP activity has also been found to increase in non-insulin-dependent diabetes mellitus (NIDDM) (Bagdade et al. 1993, Jones et al. 1996, Elchebly et al. 1996), although other researchers have found the opposite (Fielding et al. 1984, Lottenberg et al. 1996). The elevated CE transfer in NIDDM is at least partially responsible for the lower HDL-C and higher LDL+VLDL-C in these patients (Riemens et al. 1998). However, CETP mass appeared to be normal in NIDDM subjects (Jones et al. 1996). CETP activity is higher in NIDDM men with microvascular complications who smoke than in the same group of patients who do not smoke or control subjects. The increased CETP activity is at least partially responsible for the lower HDL-C/(LDL+VLDL-C) ratio in this smoking population (Dullaart et al. 1991).

In contrast to IDDM patients, acute hyperinsulemia reduced CETP activity, and this effect was correlated with baseline TG levels in NIDDM subjects (Sutherland et al. 1994, Arii et al. 1997). However, Bagdade et al. (1997) showed that, similar to IDDM patients, intraperitoneal insulin injection can normalize elevated CETPA in insulin-requiring NIDDM subjects while traditional subcutaneous injection can not. Intraperitoneal injection of insulin has been shown to reduce the exposure of extrahepatic tissues to local hyperinsulemia compared to subcutaneous injection (Bagdade et al. 1994).

The genetic polymorphisms in the CETP locus seemed to be associated with the prevalence of macrovascular complications in NIDDM. NIDDM males with EcoNI genotype 2-2 had a higher incidence of cardiovascular disease than the same group with genotype 1-1 (Ukkola et al. 1994). Unlike IDDM, Taq1B polymorphism had a significant effect on plasma CETP and HDL-C concentrations in NIDDM. Genotype B1B1 had the highest CETP and lowest HDL-C level while B2B2 had the lowest CETP and highest HDL-C (Bernard et al. 1998).

An animal study using the KKAy CETP transgenic mouse model, which is insulin-resistant and carries the CETP gene, suggested that CETP is at least partially responsible for lower HDL levels in hypertriglyceridemic NIDDM patients (Castle et al. 1998).

The role of CETP in NIDDM is not as clear as it is in IDDM patients. Nonetheless, CETP may still be partially responsible for the atherogenic lipid profiles in these subjects. The different genetypes of CETP may have different physiological roles in atherogenesis in NIDDM subjects, as also is the case in IDDM patients.

2.1.8 Mechanisms of CETP Reaction

An in vitro study using synthetic HDL particles suggested that CETP activity was affected by the fatty acid composition of CE. CETP activity increases as the acyl chain length increases in saturated fatty acids. Among all 18-carbon unsaturated fatty acids, CETP activity decreases as the degree of unsaturation increases. Docosahexanoic acid showed the greatest ability to enhance CETP activity among all fatty acids tested, while oleic acid had the second highest ability. There is no difference between human and

rabbit CETP in specificity toward various fatty acids in CE, except that rabbit CETP showed higher preference for lauric acid than human CETP (Green and Pittman 1991).

CETP mediates the exchange of cholesteryl ester in HDL and triglyceride in apo B-containing lipoproteins (Morton and Zilversmit 1983). The transfer mechanism was proposed to be either carrier-mediated (Barter and Jones, 1980) or to involve the formation of ternary complexes of HDL, apo B-containing lipoprotein, and CETP (Ihm et al. 1982). Regardless of the actual mechanism, the binding between CETP and lipoproteins is essential for the lipid exchange process. CETP interacts with lipoproteins through binding to charged groups on the lipoprotein surface (Sammett and Tall, 1985). Furthermore, it can promote the association between HDL and LDL, as apo A-I and A-II were co-precipitated with apo B after incubation with CETP. This association effect was inhibited by anti-CETP TP2 and enhanced by the presence of oleic acid. It was estimated that apo A-I was co-precipitated with apo B in the molar ratio of 3:1, whereas apo A-II had the ratio of 1:1, after 24 hours of incubation. In the presence of oleic acid, the ratio rose to 5.5: 1 and 2.3:1 for apo A-I and A-II, respectively (Lagrost and Barter 1992).

Incorporation of apo E into VLDL from apo E-deficient subject enhanced CETP activity through the increased affinity of CETP toward apo E-VLDL (Kinoshita et al. 1993). Hence, apo E may also play a role in the CETP-mediated CE transfer by promoting the interaction between CETP and VLDL.

A detailed study investigating the effects of surface charges of lipoproteins on CETP activity revealed that increases in the negative charge of LDL by acetylation or succinylation of 7% amino groups of LDL proteins resulted in the highest CE transfer between discoidal bilayer particles and modified LDL (Nishida et al. 1993). However, the

modification of the carboxyl group of LDL proteins with glycine methyl ester had little effect on CETP activity, while the modification of amino groups of HDL_3 proteins had a negative effect. The same study also suggested that HDL has a much higher affinity for CETP than LDL and VLDL, measured by the retention time of CETP in columns conjugated with various lipoproteins. It was estimated that under physiological conditions, 74, 24, and 1% of plasma CETP is bound to HDL, LDL, and VLDL, respectively. Only 1.3% of the CETP is present in the free form. Moreover, this study discovered that the ionic strength of the solution and the presence of free fatty acids or cationic detergent, such as cetyltrimethylammonium bromide (CTAB) and dodecyltrimethylammonium bromide (DTAB), also affected CETP activity. These results indicated that the hydrophobic and electrostatic interactions play important roles in the binding between lipoproteins and CETP.

Liu and Bagdade (1995) estimated that roughly 50% of the CE transferred out of HDL_3 was distributed into VLDL, while LDL and HDL_2 received, respectively, 30% and 20%. Approximately 50% of the TG transferred out of VLDL went to LDL, whereas the other 40% and 10% went to HDL_3 and HDL_2, respectively. The molar ratio of CE lost in the reaction to TG received in HDL_3 was about 3:1, which suggested the CE-TG exchange mediated by CETP may not be a equimolar pattern. The authors also discovered that CE transfer continued in VLDL-free plasma when HDL_3 did not accept any TG. Consequently, there may be two distinct and independent mechanisms mediating the transfer of CE and TG in the CETP reaction.

There is evidence that the physical state of the core lipids in LDL also plays a role in regulating the CETP reaction. CE transfer from HDL to LDL was negligible below

30°C, while it rose remarkably around body temperature (Ihm et al. 1982). Furthermore, CE transfer from synthetic liposomes to monkey LDL is temperature-dependent and can be described by two straight lines. The intersection of these two lines, inflection temperature, indicated the temperature at which the CE transfer suddenly increases (Morton and Parks, 1996). In addition, the inflection temperature is higher (41.4°C) in LDL from monkeys fed a high SFA diet than in LDL from PUFA-fed animals (32.6°C). This difference in inflection temperature may be explained by the lower fluidity of saturated fatty acyl group of CE (Tall et al. 1978, Berlin and Young Jr. 1980). This result indicated that under physiological conditions, SFA-rich CE in LDL are present in a more rigid state which is a poor substrate for CETP.

The non-esterfied fatty acids on the surface of lipoproteins, the products of lipolysis by the lipoprotein lipase reaction, can enhance CETP activity (Sammett and Tall 1985). After the reaction of lipoprotein lipase, TG-rich lipoproteins have higher affinity toward CETP (Tall et al. 1986). Moreover, CETP activity is at maximum level in the post-prandial state when lipoprotein lipase is highly active (Marcel et al. 1990). The evidence that CETP activity is impaired in lipoprotein lipase deficient subjects and can be normalized by adding exogenous lipoprotein lipase further supported this hypothesis (Bagdade et al. 1996).

The studies using deletion mutation revealed that the C-terminal amino acids 470-475 are involved in neutral lipid binding and, thus, are essential for CETP activity (Wang et al. 1995). However, this mutant still has the ability to bind with lipoproteins. This sequence is also included in the epitope of monoclonal antibody TP2, which can inhibit CETP activity (Swenson et al. 1989).

All in all, the association of CETP with its lipoprotein substrates seems to be essential for the CE transfer activity. Its activity is affected by the surface charge and the physical state of the core lipids in lipoproteins. It was suggested that the exchange of CE and TG is not limited to HDL and LDL+VLDL. CETP could also mediated the CE transfer from HDL_3 and HDL_2 and TG transfer from VLDL to LDL. As a result, the accurate measurement of CETP activity becomes a challenge.

2.1.9 CETP Gene Structure

CETP belongs to the lipid transfer protein/lipopolysaccharide binding protein gene family which also includes phospholipid transfer protein (Tall 1995). The CETP gene is composed of 16 exons (Agellon et al. 1990), and is located in the long arm of chromosome 16 (Lusis et al. 1987). Its cDNA represented a 476 amino acid hydrophobic protein and four possible N-linked glycosylation sites (Drayna et al. 1987). The difference between the molecular weight derived from cDNA, 53 kDa, and the molecular weight shown in SDS-PAGE, 66-74 kDa, is due to the posttranslational addition of glycans (Stevenson et al. 1993). CETP mRNA was detected in various human tissues, including liver, spleen, adipose tissue, small intestine, adrenal, kidney, and heart (Drayna et al. 1987). In other mammalians, adipose tissue and muscle appear to be the main expression sites of CETP mRNA (Jiang et al. 1991), while liver is the main site for CETP synthesis in primates (Quinet et al. 1991). The CETP cDNA was highly conserved among different species, including human, monkey, rabbit, and hamster, with about 80-95% homology (Tall, 1995).

The expression of human CETP in transgenic mice can be induced by a high-cholesterol diet in strains containing the natural flanking sequence (NFS) of CETP gene, but not in ones containing the metellothionein promoter (Jiang et al. 1992). The natural flanking sequence used in this study included 3.2 kb of upstream and 2.0 kb of downstream of human CETP gene. The CETP mRNA was identified in liver, spleen, small intestine, kidney, and adipose tissue, of these animals.

Further study on the promoter region of CETP gene indicated that it may contain a binding site for CCAAT/enhancer-binding protein (C/EBP), a transcription factor. The presence of C/EBP in $HepG_2$ cells enhanced CETP promoter activity, as indicated by the chloramphenicol acetyltransferase assay (Agellon et al. 1992). The tissue-specific expression of CETP is regulated by different regions within the CETP promoter. The region between -3,400 and -570 bp is essential for the expression in liver and spleen; while the sequence between -570 and -370 controls the expression in small intestine; and elements between -370 and -138 are required for adrenal expression . The sequence responsible for cholesterol induced expression was found to fall between -370 and -138 bp, which resembles the sterol regulatory element (SRE) that mediates down-regulation of the HMG-CoA reductase gene by sterol (Oliveira et al. 1996). Therefore, this sequence may also carry up-regulation activity, or another up-regulating sequence may be present in this region. This SRE-like sequence in the CETP promoter can bind to transcription factors like sterol regulatory element-binding protein-1 (SREBP-1) and Yin Yang-1 (YY-1) (Chouinard Jr et al. 1998). However, the CETP promoter with mutations in this region can still respond to cholesterol induction. Thus, another regulatory pathway may be present for the CETP gene (Chouinard Jr et al. 1998). The expression of the human CETP

gene in transgenic mice is also positively regulated by female sex steroids, as CETPA declined significantly after ovariectomy. Other ovarian factors may also be required for CETP gene expression because hormone replacement failed to restore the CETPA in ovariectomized animals (Vadlamudi et al. 1998). Furthermore, the fact that plasma CETP concentration is higher during late pregnancy in humans implied the regulatory role of female sex hormones in human CETP gene expression (Silliman et al. 1993).

Various amounts of an alternative spliced CETP mRNA which missed exon 9 was identified in different human tissues (Inazu et al. 1992). This mutated protein lacked lipid transfer ability and was poorly secreted. This mechanism of alternative splicing may regulate the expression level of the functional CETP (Inazu et al. 1992).

In summary, CETP structure is highly conserved across species. CETP is mostly synthesized in the liver, and in small amounts in adipose tissue and other organs. Its expression can be up-regulated by dietary cholesterol, possibly through a novel pathwary involving the SRE.

2.2 Reverse Cholesterol Transport

2.2.1 Overview

Reverse cholesterol transport (RCT), a process that transfers cholesterol from extrahepatic tissues to liver for excretion or redistribution, plays an important role in cholesterol metabolism. As illustrated in Figure 2.1, the process is composed of four major steps: (1) cholesterol efflux from peripheral tissues into HDL; (2) cholesterol esterification in HDL by LCAT; (3) CE transfer from HDL to apo B-containing lipoproteins in exchange for TG; (4) hepatic uptake of CE in apo B-containing

lipoproteins through LDL receptor (indirect pathway) or CE in HDL through the reaction of hepatic lipase (direct pathway) (Hill and McQueen 1997).

2.2.2 Cholesterol Efflux from Peripheral Tissues

The majority of cholesterol efflux from peripheral tissues is first taken up by prebeta-1-HDL, a small lipid-poor particle that contains apo A-I (Castro and Fielding 1988). Two mechanisms of cholesterol transfer from extrahepatic tissues into HDL have been proposed. The first one is through diffusion, where albumin may serve as the carrier to facilitate cholesterol transfer (Fielding and Fielding 1995). The other mechanism is a receptor-mediated, protein kinase C-required transfer. Several receptors for apolipoproteins in HDL have been identified on the surface of various peripheral cells (Oram et al. 1987, Barbaras et al. 1990). After binding of HDL with the receptor, cellular cholesterol is transferred to the plasma membrane and subsequently to HDL through a protein kinase C-mediated pathway (Mendez et al. 1991). This mechanism relies on the free cholesterol gradient between HDL and the intracellular pool; therefore, it is LCAT-dependent (Huang et al. 1993). This receptor-mediated mechanism, although faster than diffusion, was only seen cholesterol-loaded cells (Oram et al. 1991).

2.2.3 Cholesterol Esterification in HDL

The LCAT reaction is the major source of HDL-CE in humans (Skipski 1972). The free cholesterol taken up by HDL is subsequently esterified by LCAT to prevent it from diffusing out of the lipoprotein particle. The esterification also reduces the free cholesterol pool within HDL, possibly maintaining the free cholesterol gradient favoring

cholesterol uptake by the lipoprotein. LCAT plays a role in the maturation and enlargement of HDL. When HDL continues to take up cholesterol, it transforms from a small lipid-poor particle, prebeta-1 HDL, to a discoidal prebeta-2, and eventually a spherical CE-rich particle HDL_2 and HDL_3. LCAT is associated with HDL particles from the prebeta-2 to HDL_2 stage (Hill and McQueen 1997), while the affinity for LCAT decreases as the HDL particle size increases (Park et al. 1987).

Several subjects with LCAT deficiency, resulting from various genetic mutations, have been found to suffer from premature CVD (Kuivenhoven et al. 1995 and 1996). This discovery suggested the importance of LCAT in antiatherogenesis, although many other LCAT deficient subjects showed no signs of premature CVD. Other abnormal lipid profiles were also present in LCAT deficient subjects, including high levels of nascent HDL (Norum et al. 1975) and high cholesterol content in erythrocyte membranes (Glomset JA et al. 1983).

2.2.4 Cholesterol Transport by CETP

CETP facilitates the transfer of CE from HDL into apo B-containing lipoproteins which are further metabolized through the hepatic LDL receptor. Moreover, CETP enhances cholesterol esterification by elevating LCAT activity (Channol et al. 1990, Jones et al. 1996), which may stimulate the cellular cholesterol efflux. Furthermore, CETP was demonstrated to facilitate the conversion of pre-beta-HDL from alpha-HDL, while LCAT is responsible for generating alpha-HDL from pre-beta-HDL (Kunitake et al. 1992). Therefore, CETP is responsible for regenerating small HDL particles that are optimal for uptake of extrahepatic cellular cholesterol. CETP can also transform HDL_3

into two types of particles larger and smaller in size than the original lipoprotein (Lagrost et al. 1990), suggesting that CETP participates in HDL remodeling.

2.2.5 Hepatic Lipase-Mediated Cholesterol Uptake by the Liver

Hepatic lipase is synthesized and secreted by the liver (Rea et al. 1993) and is present on the surface of hepatocytes and liver sinusoidal endothelial cells (Sanan et al. 1997). It catalyzes the conversion of HDL_2 to HDL_3 through hydrolysis of TG in the HDL_2 lipid core (Fan et al. 1994). Overexpression of hepatic lipase in rabbits caused a marked reduction of large HDL particles, HDL_1 and HDL_2, with moderate decrease in dense HDL_3 and IDL (Fan et al. 1994).

Hepatic lipase facilitates the CE release from TG-rich HDL_2; the CE is subsequently taken up by the liver. This process is dependent on the phospholipase A1 activity of hepatic lipase, but not its triglyceride hydrolysis activity (Marques-Vidal et al. 1994). The hepatic uptake of CE mediated by hepatic lipase is apparently receptor-independent. Other receptor-mediated pathways may also be present as HDL binding proteins have been identified in livers of humans (Murao et al. 1997, Matsumoto et al. 1997), rodents (Acton et al. 1996), and pigs (de Crom et al. 1992). One study investigating the mechanism of hepatic uptake of HDL showed that the HDL binding protein can accumulate HDL-derived cholesterol in the cell without internalization or break down of apolipoprotein (Murao et al. 1997).

2.2.6 Effects of Dietary Fatty Acids on LCAT Activity

Two human studies revealed that diets high in PUFAs decreased LCAT activity, compared to SFAs (Gjone et al. 1972, Miller et al. 1975). Gjone et al. (1972) discovered that after 3 weeks, a diet containing 40 en% from soybean oil (high in 18:2) significantly reduced LCAT activity, while LCAT activity remained constant in the group consuming the same amount of oleum vegetable tenue (rich in MCT). However, the plasma TC/CE ratio did not change in either diet group. Thus, other factors may also be involved in regulating plasma cholesterol esterification. Similar results were also observed by Miller et al. (1975).

Baudet et al. (1988) investigated the effects of individual fatty acids on LCAT activity. Six weeks after human subjects consumed 30 en% from four different sources of fats, LCAT increased in the sunflower (P/S = 1.68) and peanut oil (P/S = 6.31) groups, but decreased in the low erucic acid rapeseed oil (P/S = 4.17) and milk fat (P/S = 0.10) groups. Thus, LCAT activity seemed to be unrelated with the ratio of dietary PUFAs to SFAs. Instead, the authors discovered that LCAT activity correlated positively with the percentage of linoleic acid in serum phospholipids and cholesteryl esters, while negatively with the percentage of oleic acid in the same fractions. The lack of correlation between LCAT activity and plasma TC/CE ratio reported by this group also hinted that the distribution of HDL subfractions was regulated by other factor(s). A later study demonstrated that diet had a significant impact on fatty acid compositions of HDL-PC, and HDL rich in n-3-PUFA-PC resulted in lower LCAT activity than HDL-PC high in n-6-PUFA, MUFAs, or SFAs (Thornburg et al. 1995). Another human study also supported that a high-fish-oil diet reduced LCAT activity (Abbey et al. 1990).

A baboon study (Mott et al. 1987) investigating the effects of dietary fat and cholesterol on LCAT activity showed that a high-cholesterol diet lowered fractional cholesterol esterification rate, compared to a low-cholesterol group, while molar cholesterol esterification rate remained similar in both groups. These differences resulted from the high free cholesterol concentration in the high-cholesterol group, which was multiplied by fractional esterification rate to obtain the molar rate. Furthermore, SFAs increased molar esterification rate compared to PUFAs, while no change was observed in fractional esterification rate.

Several in vitro studies using recombinant HDL containing phosphotidylcholine (PC) with various fatty acid components have been conducted to investigate the substrate preference of LCAT. Dobiasova (1983) estimated that the order of relative transesterification rates was 18:2>20:4=14:0>18:1>10:0=12:0>8:0>16:0>18:3=18:0. Parks and Gebre (1997) using PC containing 16:0 in the sn-1 position and various unsaturated fatty acids in the sn-2 position showed the order of LCAT Vmax as 18:1>20:5>20:4>22:6. This study also reported lower activation energy of the LCAT reaction in PC containing PUFA than in PC containing 18:1, which was the opposite to what would be expected from Vmax data.

Another study using recombinant PC suggested the rank of LCAT preference was 18:2>18:1>20:4>16:0>20:5+22:6 (Subbaiah and Liu, 1996). The same study also revealed that the 14 vertebrates studied could be separated into two groups according to their LCAT specificities. LCAT of group 1, including atherosclerosis-prone species such as rabbit, pig, guinea pig, baboon, human, and hamster, showed preference for 18:2 in the

sn-2 position of PC. Whereas LCAT of group 2, including atherosclerosis-resistant species like rat, and mouse, preferred 20:4 in the sn-2 position.

2.2.7 Effects of Dietary Fatty Acids on CETP Activity

Similar to LCAT, CETP activity appears to be affected by dietary fatty acid composition. Groener et al. (1991) reported that a diet high in oleic acid significantly reduced CETP activity, compared to a high-SFA baseline diet. On the other hand, a diet high in linoleic acid only resulted in a slight and nonsignificant reduction in CETP activity. A positive correlation was found between changes in CETP activity and changes in TC and (LDL+VLDL)-C levels. Different SFAs also had distinct effect on CETP activity. When substituted for 5 en% of MUFA in the diet, palmitic acid increased CETP activity from baseline, while stearic acid showed no effect on CETP activity (Schwab et al. 1996). The palmitic acid group also had higher CETP activity than the stearic acid group. However, HDL-C was also higher in the palmitic acid group than in the stearic acid group. Cox et al. (1995) examined three different experimental diets containing 18 en% from coconut oil (rich in 12:0 and 14:0), butter (rich in 14:0 and 16:0), or safflower oil (rich in 18:2). CETP activity was the highest on butter, followed by coconut, and lowest on the safflower oil diet. Plasma TC and LDL-C were in the same order as CETP activity.

Another study revealed that fish oil had a more profound effect on decreasing CETP activity than linoleic and alpha-linolenic acids, possibly because of the lower plasma and VLDL-TG observed in the fish oil group (Abbey et al. 1990). Trans fatty acids, such as elaidic acid (trans-18:1), enhanced CETP activity compared to oleic acid

(Abbey and Nestel 1994) and linoleic and stearic acid (van Tol et al. 1995), thereby decreasing HDL-C.

To sum up, RCT is an important pathway regulating the cholesterol metabolism in the whole body. It prevents cholesterol accumulation in peripheral tissues by transferring it to liver for excretion or biosynthesis of other compounds. Various dietary fatty acids may have distinct effects on RCT because the enzymes involved in the process have different affinity for these acids.

2.3 Cholesterolemic Effects of Dietary Fatty Acids

2.3.1 Effects of Dietary Fatty Acids on Plasma Cholesterol

It has been demonstrated that different dietary fatty acids have distinct effects on plasma TC, HDL-C, and LDL-C levels. More than 30 years ago, several equations were derived from meta-analysis of reported data to quantify the effects of dietary fatty acids on plasma total cholesterol concentrations (Keys et al. 1957, Keys et al. 1965a, Hegsted et al. 1965). More recent studies have extended the equations to the changes in HDL-C and LDL-C (Mensink and Katan 1992, Hegsted et al. 1993, Yu et al. 1995). Table 2.2 shows the association of dietary fatty acid with the change in plasma total cholesterol, while Table 2.3 shows the equations predicting changes in HDL-C and LDL-C.

2.3.1.1 Saturated Fatty Acids

All the equations mentioned above agreed that SFAs significantly increased plasma TC, HDL-C, and LDL-C. However, when the effects of individual saturated fatty acids were further investigated, Yu et al. (1995) suggested that stearic acid has a neutral effect on any of these three cholesterol concentrations. This result agreed with the earlier

equations (Hegsted et al. 1965, Keys et al. 1965, Mensink and Katan 1992) that showed stearic acid, unlike other SFAs, had no effect on plasma cholesterol concentrations. Some individual studies even suggested that stearic acid could lower TC, HDL-C and LDL-C (Bonanome and Grundy, 1988, Denke and Grundy 1991, Derr et al. 1993), compared to diets high in palmitic acid or butter. On the other hand, lauric acid, myristic acid, and palmitic acid increased all three cholesterol parameters in these equations.

2.3.1.2 PUFAs and MUFAs

MUFAs have been shown to have neutral (Mensink and Katan, 1992, Hegsted et al. 1993) or hypocholesterolemic, (Yu et al. 1995) effects on TC and LDL-C. PUFAs were widely considered by these meta-analyses to be able to lower TC and LDL-C. Furthermore, MUFAs did not reduce TC and LDL-C as much as PUFA did, as indicated by the regression coefficients. On the contrary, several researchers found that MUFAs had a hypocholesterolemic effect similar to PUFAs' (Mattson and Grundy 1985, Mata et al. 1992).

The effects of PUFAs and MUFAs on HDL-C are less consistent among these equations. Hegsted et al. (1993) failed to find a equation explaining more than 40% of the variance of HDL-C. Mensink and Katan (1992) and Yu et al. (1995) all agreed that both PUFAs and MUFAs can increase HDL-C when substituted for carbohydrate in the diet, while MUFAs are more potent, although some studies showed that MUFAs had no effect on HDL-C concentration (Gainsberg et al. 1990, Berry et al. 1992).

2.3.1.3 Trans Fatty Acids

Many studies have attempted to determine the effect of trans fatty acids, mainly elaidic acid (trans C18:1w9), on plasma cholesterol levels. However, these studies replaced linoleate or oleate with trans fatty acids (Mensink et al. 1990, Nestel et al. 1993, Lichtenstein et al. 1993, Judd et al. 1994). From the equations mentioned above, both linoleate and oleate had potential hypocholesterolemic effects. Therefore, the observed hypercholesterolemic effects of trans fatty acids in these studies were difficult to distinguish from the effect of the decreased dietary MUFAs and PUFAs. In one study, Zock and Katan (1992) reported that elaidic acid had a similar effect as stearic acid. Stearic acid is widely considered to be neutral on plasma cholesterol concentrations. As a result, trans fatty acids may not be as hypercholesterolemic as most studies suggested. The epidemiological studies of trans fatty acid intake and incidence of CVD produced controversial results. A large scale study in Europeans failed to show a link between the incidence of MI and trans 18:1 intake, as reflected by the fatty acid content in adipose tissue (Aro et al. 1995), while the other study even suggested a negative correlation between these two factors (Roberts et al. 1995). On the other hand, other studies suggested that trans fatty acid may be positively related to CVD (Booyens et al. 1988, Kromhout et al. 1995).

2.3.3.4 Interactions with Other Variables

When discussing cholesterolemic effects of dietary fats, it is important to consider the cholesterol intake, since dietary cholesterol is also a significant determinant of plasma cholesterol levels (Keys et al. 1965a, Hegsted et al. 1965, Hegsted et al. 1993).

The interactions among various dietary fatty acids may also deserve attention. Yu et al. (1995) suggested that, when hypercholesterolemic SFAs are low in the diet, the hypocholesterolemic effect of MUFAs becomes substantial; whereas when SFAs are high, they may mask the hypocholesterolemic effect of MUFAs.

Furthermore, individual differences in cholesterol metabolism also play a role in response to dietary fatty acids. It has been reported that hypercholesterolemic subjects may be more responsive to dietary change than normocholesterolemic ones (Keys et al. 1959, Keys et al. 1965b). Moreover, there might be gender differences in the response to dietary fatty acid changes. For example, PUFA reduced TC and LDL in men but not in women, while stearic acid lowered HDL-C in women but not in men (Yu et al. 1995). On the contrary, our study showed that PUFA can decrease TC and LDL-C in women (Park and Snook, 1995).

In addition, genetic background may also contribute to the changes in plasma cholesterol concentrations. Apo E polymorphism has been suggested to play a role in lipid metabolism and may affect the response to dietary fats (Gregg et al. 1986, Martin et al. 1993). CETP polymorphism also may influence dietary-induced HDL-C changes in type 1 diabetic patients (Dullaart et al. 1997).

2.3.4 Mechanisms Mediating Cholesterolemic Effects of Fatty Acids

The dietary-induced changes in LDL-C mostly result from alterations in hepatic LDL-receptor (LDLr) activity (J^m), and generation rate of VLDL (J_t). The interactions among these factors are shown in Figure 2.2. Hepatic LDLr expression is regulated by the sterol regulatory element-binding protein-1 (SREBP-1), a transcription factor located

intrinsically to membranes of the nuclear envelope and endoplasmic reticulum (Sato et al. 1994). The biological activity of SREBP-1 responds to the regulatory cholesterol pool (C^R) in hepatocytes. In cholesterol-depleted cells this transcription factor is hydrolyzed by a specific protease, and the N-terminal fragment enters the nucleus and activates the transcription of the LDLr gene by binding to the sterol regulatory element-1 (SRE-1). Excess cholesterol in C^R blocked the transcriptional activation by inhibiting the proteolytic cleavage.

It has been suspected that C^R is mainly regulated by hepatic acyl-CoA:cholesterol acyltransferase (ACAT) which removes cholesterol from C^R by esterification (Dietschy 1998). Its activity is supposed to be affected by both of the substrates, cholesterol and fatty acids. When dietary cholesterol intake is the only variable, the inverse relationship between LDLr activity and hepatic CE concentration has been discovered by several researchers (Daumerie et al. 1992, Woollett et al. 1992, Spady et al. 1993). This inverse relationship indicates that, when the entry of cholesterol into the liver increases, LDLr activity is suppressed and more cholesterol was esterified into the storage form, CE. However, when dietary fatty acid composition changes while dietary cholesterol level stays constant, the LDLr activity and liver CE related positively (Daumerie et al. 1992). In other word, when the cholesterol entry into the liver remains unchanged, the more cholesterol is removed from C^R by esterification, the higher the LDLr activity is.

The effects of dietary fatty acids were suggested to be mainly redistribution of the cholesterol pool in hepatocytes. Two studies, one in humans (Fielding et al. 1995) and one in hamsters (spady and Dietschy 1988) revealed that increasing dietary cholesterol alone can increase LDL-C, while the hypercholesterolemic effect of SFAs became more

potent with higher cholesterol intake (Figure 2.3). The substrate preference of ACAT toward various fatty acids may be the primary mechanism of their cholesterolemic effects. The LDL-C-elevating lauric acid, myristic acid, and palmitic acid inhibited cholesterol esterification, and presumably expanded the C^R pool (Daumerie et al. 1992). Therefore, LDLr mRNA decreased and LDLr activity (J^m) was suppressed. These fatty acids also increased J_t. Conversely, LDL-C-lowering oleic acid, the preferred substrate for ACAT, shifted cholesterol from C^R into CE pool. Therefore, LDLr expression increased and its activity J^m increased, while J_t decreased (Daumerie et al. 1992, Spady et al. 1993). Linoleic acid had a similar but less potent effect compared to oleic acid on LDL-C levels (Spady et al. 1993). Other fatty acids that had a neutral effect on LDL-C concentration, including butanoic acid (4:0), caproic acid (6:0), caprylic acid (8:0), capric acid (10:0), stearic acid, and elaidic acid, did not significantly affect any parameter in LDL metabolism (Spady et al. 1993). Figure 2.4 summarizes the interactions among dietary fatty acids and cholesterol regulating hepatic LDLR activity.

In summary, SFAs, except 18:0 tend to increase plasma TC, HDL-C, and LDL-C, while 18:0 is considered to be neutral. On the other hand, MUFAs and PUFAs are thought be to hypocholesterolemic. The effects of trans fatty acids were mixed in various studies and may require further investigation. The dietary cholesterol intake and genetic differences also contribute to the determination of plasma lipoprotein profiles. The major mechanism through which dietary fatty acids and cholesterol regulate plasma cholesterol levels is the homeostasis across the liver. The combined effects of J^m and J_t determine the plasma LDL level. The free cholesterol regulatory pool C^R, regulated by ACAT activity, controls the LDLR activity.

2.4 Transgenic and knockout mice models used in studies of atherosclerosis

The mouse is a suitable model for the study of atherogenesis because of the presence of several inbred stains with well-defined genetic backgrounds, smaller body size, and shorter life span. Several strains susceptible to dietary-induced atherosclerosis such as C57BL/6, and resistant strains like CBA/J, have been developed (Paigen et al. 1990). The studies using strains with various susceptibility and their crossover offsprings identified several genes responsible for susceptibility of atherosclerosis, including *Ath-1, Ath-2, and Ath-3* (Paigen et al. 1987, 1989, Stewart-Phillips et al. 1989). The development of various lines of transgenic and knockout mice further provides the models for studying the effect of individual genes in atherosclerosis. Several commercially available mice models are introduced below and summarized in Table 2.4.

The many studies using CETP transgenic mice were described in 2.1.2. The coexpression of CETP and apo B genes produced a plasma HDL/LDL distribution similar to humans (Grass et al. 1995). The expression of human apo A-I gene resulted in two fractions of HDL particles with sizes similar to human HDL_{2b} and HDL_{3a}, rather than the homogenous HDL seen in controls (Rubin et al. 1991). This result suggested the important role of apo A-I in HDL size distribution. On the other hand, the apo A-I knockout mice, which did not express any apo A-I, had significantly lower plasma cholesterol and HDL levels than wild type animals (Williamson et al. 1992). The overexpression of mouse apo A-II resulted in higher HDL-C, LDL-C, and VLDL-C, as well as larger HDL particles (Hedrick et al. 1993). However, the transgenic mice

expressing human apo A-II gene had smaller HDL particles containing human apo A-II, while HDL-C levels were similar to wild type animals (Schultz et al. 1992).

The mice expressing mutated apo B gene produced a defective apo B protein, apo B70. This train of mouse had typical characteristics of human hypobetalipoproteinemia, including decreased plasma TC, LDL+VLDL-C, HDL-C, and TG. Unexpectedly, these mice also had higher incidence of exencephal and hydrocephaly (Homanics et al. 1993). Using a targeted mutagenesis technique, mice producing only apo B48 or apo B100 were produced. Compared to wild type mice consuming regular chow, mice expressing only apo B48 had higher LDL-C, LDL-TG and VLDL-TG, while these parameters were lower in mice expressing only apo B100 (Farese et al. 1996). After crossover with apo E-deficient mice, apo B48-E-deficient mice showed more severe atherosclerotic lesions, while apo B100-E-deficient mice showed less severe lesions, compared to apo B-normal-E-deficient mice. However, the severity of lesions was only correlated with plasma cholesterol concentrations, but not apo B phenotypes (Veniant et al. 1997).

The mice unable to produce apo E showed 4-fold increase in plasma TC and severe atherosclerotic lesions in aortas (Zhang et al. 1992). The knockout mice lacking LDL receptor had marked increased IDL-C and LDL-C, resulting in higher TC. The clearance of IDL and LDL were significantly prolonged, compared to wild type animals. The HDL-C levels and clearance rate were similar to wild type mice (Ishibashi et al. 1993). The mice lacking both apo E and LDL receptor had plasma lipid profile similar to apo E-deficient mice (Ishibashi et al. 1994).

Transgenic mice overexpressing human apo CI gene had impaired clearance of VLDL and lipoprotein remnants, resulting in elevated plasma TG, IDL, LDL, and VLDL

levels (Schachter et al. 1996). This strain could be used as a model of decreased postprandial clearance of TG-rich lipoproteins and their remnants. Apo CII transgenic mice may serve as a hypertriglyceridemic model as the clearance of TG-rich VLDL was delayed (Shachter et al. 1994). The knockout mice unable to express apo CIII had lower plasma TG than the wild type. These apo CIII-deficient mice also showed postprandial hypotriglyceridemia resulting from accelerated clearance of chylomicrons (Maeda et al. 1994).

In summary, the advance in genetic techniques has allowed researchers to study the effect of specific gene(s). However, care must be taken in interpreting data obtained from these animal models. The factors which should be taken into consideration may include (1) whether the gene is expressed in the physiological level, (2) whether the gene expression is under physiological control, and (3) the interactions among the transgene or knockout gene and others.

Location	Type	Base Pair	Codon	Phenotype
Exon 1	Deletion	C294	38 ala→stop	↑HDL
Exon 9	Alternative splice			exon 9 deleted reduced SA[a] regulatory role?
Exon 10	Nonsense	C1106T	309 gln→stop	↓mRNA production inactive protein
Exon 11	Missense	G1298G	373 ala→pro	↑HDL
Exon 12	Missense	A1394G	405 ile→val	↑HDL
Intron 14	Substitution	g+1a		splice defect in mRNA production SA[a] unaffected ↑HDL
Intron 14	Insertion	t+3		splice defect in mRNA production
Exon 15	Missense	A1506G	442 asp→gly	SA[a] reduced ↑HDL dominant effect?
Exon 15	Missense	G1533A	451 arg→gln	↑HDL
Exon 16	Missense	G1996A	3' untranslated region	↓CETP activity in plasma no lipid changes

Table 2.1: Mutations in cholesteryl ester transfer protein. (Hill and McQueen 1997). [a] SA, specific activity.

	Coefficient for ΔS	Coefficient for ΔM	Coefficient for ΔP	Coefficient for ΔC	Reference
ΔTC=	2.74		-1.31		Keys et al. 1957
ΔTC=	2.40[a]		-1.20	1.5[b]	Keys et al. 1965
ΔTC=	2.16		-1.65	0.065	Hegsted et al. 1965
ΔTC=	2.74[a]		-1.83	0.071	Hegsted et al. 1965
ΔTC=	2.16[a]	-0.12	-0.60		Mensink et al. 1992
ΔTC=	2.10		-1.16	0.067	Hegsted et al. 1993
ΔTC=	2.02[a]	-0.48	-0.96		Yu et al. 1995

Table 2.2: Regression coefficients of equations predicting the effects of dietary fatty acids on serum total cholesterol levels. ΔTC, changes in total cholesterol in mg/dl; ΔS, changes in %en for all SFAs; ΔM, changes in %en for MUFAs; ΔP, changes in %en for MUFAs; ΔC, changes in dietary cholesterol in mg/1000 kcal. [a]Changes in %en for 12:0, 14:0, and 16:0 only; [b]coefficient for $\Delta C^{1/2}$.

	Coefficient for ΔS	Coefficient for ΔM	Coefficient for ΔP	Coefficient for ΔC	Reference
ΔLDL-C=	1.83[a]	-0.24	-0.55		Mensink et al. 1992
ΔLDL-C=	1.74		-0.77	0.044	Hegsted et al. 1993
ΔLDL-C=	1.46[a]	-0.69	-0.96		Yu et al. 1995
ΔHDL-C=	0.67[a]	0.34	0.28		Mensink et al. 1992
ΔHDL-C=	0.62[a]	0.39	0.24		Yu et al. 1995

Table 2.3: Regression coefficients of equations predicting the effects of dietary fatty acids on serum LDL- and HDL-cholesterol levels. ΔLDL-C, changes in LDL-cholesterol in mg/dl; ΔHDL-C, changes in HDL-cholesterol in mg/dl; ΔS, changes in %en for all SFAs; ΔM, changes in %en for MUFAs; ΔP, changes in %en for MUFAs; ΔC, changes in dietary cholesterol in mg/1000 kcal. [a]Changes in %en for 12:0, 14:0, and 16:0 only.

Targeted genes[a]	Phenotypes[b]	References

+ h-CETP	↓HDL-C, ↓TC, ↓LDL-R mRNA	Agellon et al. 1991
+ h-CETP, + h-apo A-I	↓↓HDL-C, ↓TC, vs +h-apo A-I mice	Hayek et al. 1992
+ h-CETP, + h-apo B	Lipd profile similar to humans	Grass et al. 1995
+ h-apo A-I	2 HDL subclasses similar to humans	Rubin et al. 1991
- m-apo A-I	↓HDL-C, ↓TC	Williamson et al. 1992
+ h-apo A-II	↓HDL particle size, –HDL-C	Schultz et al. 1992
+ m-apo A-II	↑HDL-C, ↑HDL particle size, ↑LDL+VLDL-C	Hedrick et al. 1993
- m-apo B	hypobetalipoproteinemia, ↓TC, ↓HDL-C, ↓LDL+VLDL-C, ↓TG	Homanics et al. 1993
m-apo B48 only	↑LDL-C, ↑LDL-TG, ↑VLDL-TG	Farese et al. 1996
m-apo B100 only	↓LDL-C, ↓LDL-TG, ↓VLDL-TG	Farese et al. 1996
- m-apo E, m-apo B48 only	↑TC, ↑aorta lesions vs -m-apo E mice	Veniant et al. 1997
- m-apo E, m-apo B100 only	↓TC, ↓aorta lesions vs -m-apo E mice	Veniant et al. 1997
+ h-apo CI	↑TG, ↑VLDL-C, ↑IDL-C, ↓VLDL remnant clearance	Shachter et al. 1996
+ h-apo CII	↑TG, ↑VLDL-C, ↓VLDL remnant clearance	Schatcher et al. 1994
- m-apo CIII	↓TG, ↑chylomicron clearance	Maeda et al. 1994
- m-apo E	↑TC, ↑IDL-C, ↑LDL-C, ↓clearance of LDL and VLDL, ↑arterial lesions	Zhang et al. 1992 Ishibashi et al. 1994
- m-apo E, - m-LDL receptor	similar to - m-apo E mice	Zhang et al. 1992 Ishibashi et al. 1994

Table 2.4: Summary of commercially available transgenic and knockout mice models for cardiovascular research. [a] +: addition of the gene, -: removal of the gene, h: human, m: mouse; [b] compared to wild type mice if not mentioned otherwise, ↓: decrease, ↑: increase, –: no change.

Figure 2.1: Overview of reverse cholesterol transport. HL, hepatic lipase; FA, unesterified fatty acids; HDL-R, HDL receptor; PL, phospholipid (Hill and McQueen 1997).

Figure 2.2: A model for the major steps that regulate steady-state concentrations of LDL-C (Spady et al. 1993).

Figure 2.3: Cholesterolemic effects of fatty acids are dependent on the level of dietary cholesterol intake. This figure shows the absolute concentration of LDL-C achieved in hamsters or humans fed predominantly saturated fatty acids (SFA) or unsaturated fatty acids (USFA) under circumstances in which the amount of cholesterol in the diet was varied. The x-axis represented the dietary cholesterol levels relative to the daily cholesterol synthesis in these two species (Dietschy 1998).

Figure 2.4: The interactions among dietary fatty acids and cholesterol regulate hepatic LDL receptor activity. The insert shows the mechanisms involved in this regulation (Spady et al. 1993).

CHAPTER 3

SUBJECTS AND METHODS

3.1 Study Design

This dissertation is composed of two studies using type 1 diabetic children and CETP transgenic mice as experimental models. We first developed an enzyme-linked immunosorbent assay (ELISA) for plasma CETP concentration. This assay was subsequently used in both human and animal studies. The data on plasma glucose, glycosylated hemoglobulin, apolipoprotein, and lipoprotein cholesterol concentrations in diabetic subjects were obtained from previous studies in our laboratory.

3.2 Human Study

3.2.1 Subjects

The human study protocol was approved by both Columbus Children's Hospital and The Ohio State University. Thirty-five type 1 diabetic children (12 males and 23 females, ages 5-12) were recruited by personnel at Columbus Children's Hospital. After the nature of the procedure was explained, a parent or guardian signed an informed consent statement approved by both Columbus Children's Hospital Institutional Review Board for

Human Studies and The Ohio State University Institutional Review Board for Human Studies. Blood samples were withdrawn by venipuncture from each subject after an overnight fast.

3.2.2 Plasma CETP concentration

3.2.2.1 Buffers and Solutions

Coating buffer contained 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6. Saturation buffer contained phosphate-buffered saline (PBS), 1% (w/v) skim milk. Working buffer contained PBS, 1% (w/v) skim milk, 0.1% (v/v) Triton X-100. Washing buffer contained PBS, 0.5% (v/v) Tween 20.

3.2.2.2 Biotinylation of TP2

Monoclonal anti-CETP TP2 and TP20 were purchased from the Ottawa Heart Institute (Ontario, Canada). Biotinylated-TP2 was prepared using a EZ-Link biotinylation kit from Pierce (Rockford, IL), performed according to the recommendations from the manufacturer. 20 μl PBS containing sulfo-NHS-LC-biotin was added into 250 μl PBS containing 500 μg TP2. The mixture was incubated at room temperature for 30 min, then dialyzed against PBS at 4^{o}C overnight. It was adjusted to 0.1 mg/ml with PBS, separated into small aliquots, and stored at -70^{o}C until use.

3.2.2.3 ELISA Procedure

Polystyrene 96-well microtiter plates were used as a solid phase. 200 μl TP20 in coating buffer (1ng/ μl) was added to each well, and the plates were incubated at 37^{o}C for 1.5 hr. After removing unbound TP20, 200 μl saturation buffer was added, and the plates were incubated at 37^{o}C for 1 hr. The wells were rinsed with 250 μl PBS twice before adding in triplicate 100 μl 1:7 diluted plasma from type 1 diabetic subjects and reference plasma (pooled plasma from 4 healthy donors) in working buffer. The diluted plasma was incubated at 37^{o}C for 1 hr prior to adding to the wells. 3 wells containing only working buffer served as blanks. The plates were incubated at room temperature for 3.5 hr. The wells were washed 5 times with 250 μl washing buffer and rinsed with 250 μl PBS twice. 200 μl biotin-TP2 in working buffer (0.5 ng/ μl) was added to each well. The plates were incubated at 4^{o}C overnight. The wells were washed with 250 μl washing buffer 5 times and rinsed with 250 μl PBS twice. To each well, 200 μl horseradish peroxidase-conjugated avidin (a 1:5000 dilution of Sigma stock prepared using working buffer) was added. The plates were incubated at room temperature for 2 hr. The wells were washed 5 times with 250 μl washing buffer and rinsed with 250 μl PBS twice. 150 μl Turbo-TMB substrate solution was added and the plates were incubated at room temperature for 30 min. 100 μl 1M H_2SO_4 was then added to stop the enzyme reaction. The plates were read at 450 nm with a Spectra Max 250 microtiter plate reader from Molecular Devices (Sunnyvale, CA). The absorbances of samples from type 1 diabetic subjects were compared to that of reference plasma, and expressed as 'ng biotin-TP2 bound per μl plasma' (see below).

3.2.2.4 Construction of Standard Curve and Calibration of Reference Plasma

A curve of absorbance of known amounts of biotin-TP2 bound directly onto the microtiter plate was constructed. To do this, 200 μl serial diluted biotin-TP2 in coating buffer (6.25, 12.5, 25, 50, 100 ng/200 μl) was added in triplicate. On the same plate, 200 μl TP20 in coating buffer (1ng/ μl) was also added to 6 other wells, and the plates were incubated at 37^{o}C for 1.5 hr. The unbound TP20 and biotin-TP2 solution was then removed, 200 μl saturation buffer was added, and the plate was incubated at 37^{o}C for 1 hr. The wells were rinsed with 250 μl PBS twice. After incubating at 37^{o}C for 1 hr, 100 μl 1:7 diluted reference plasma in working buffer was added into 6 wells coated with TP20. The wells coated with biotin-TP2 were covered with 250 μl PBS. The plate was incubated at 37^{o}C for 3.5 hr. The wells containing reference plasma were washed, incubated with biotin-TP2 overnight at 4^{o}C, and then washed, as described above. PBS was then removed from wells directly coated with biotin-TP2. 200 μl 1:5000 diluted horseradish peroxidase-conjugated avidin in working buffer was added into all wells. The plate was then incubated, washed, color-developed, and read as described above. Absorbance was plotted as a function of amount of biotin-TP2 coated directly onto the wells, using a linear regression program. The absorbance units of reference plasma, after fitting to the regression equation, were converted to the amount of biotin-TP2 bound.

3.2.3 Fasting Blood Glucose and Glycosylated Hemoglobin

Fasting blood glucose was measured with an enzymatic colorimetric kit containing hexokinase and glucose-6-phosphate dehydrogenase (Sigma). Total glycosylated

hemoglobin and glycosylated hemoglobin A1c were measured with colorimetric methods following the separation by affinity resin column (Sigma).

3.2.4 Plasma lipids and lipoproteins analyses

Plasma total cholesterol and TG were measured enzymatically (Stanbio Laboratory, San Antonio, TX). HDL-C was determined after precipitating apo B-containing lipoproteins from plasma. LDL-C and VLDL-C were then calculated by the Friedwald equation (Friedwald et al. 1972). Plasma free cholesterol was measured according to Deacon and Dawson (1979) with slight modification. 10 μl plasma was incubated at 37^{o}C for 20 min with 1 ml reagent containing 3 mM sodium cholate, 0.82 mM 4-aminoantipyrine, 14 mM phenol, 50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 0.17 mM Carbowax-6000, 300 U/L cholesterol oxidase, and 1000 U/L horseradish peroxidase. The absorbances were read at 500 nm. Plasma lipoproteins were isolated by a single-spin density gradient ultracentrifugation method according to Havel et al (1955) and Terpstra et al. (1981) with minor adjustment. Briefly, 2 ml fresh plasma was adjusted to 1.25 g/ml by adding KBr and sucrose, followed by overlaying with 2 ml 1.225 g/ml KBr, 4 ml 1.10 g/ml KBr, and 4 ml deionized water. After centrifugation at 200,000 g at 20^{o}C for 24 hrs, fractions of VLDL, LDL, HDL_2, and HDL_3 were removed by aspiration. LDL-FC, LDL-TG, VLDL-TG, HDL_2-C, and HDL_3-C were measured with commercial kits (Sigma). The concentration of LDL-CE was estimated by the difference between LDL-C and LDL-FC.

3.2.5 CETP and LCAT Activities

Plasma CETP and LCAT activities were measured in triplicate according to Channon et al (1990). 50 μl of [^{3}H]cholesterol-albumin emulsion (0.3 μCi) was added into 500 μl plasma. The mixture was incubated at 37^{o}C for 3 hr. For CETP activity, the radioactivity of a 50 μl aliquot was measured by a liquid scintillation counter (Beckman, Palo Alto, CA). LDL+VLDL in a 300 μl aliquot was precipitated by 60 μl 4% (w/v) sodium phosphotungstate in 0.5 M $MgCl_2$. After centrifugation, the supernatant HDL was removed and the radioactivity in 100 μl aliquot was measured. The pellet was washed twice. The lipid was extracted from the pellet with chloroform/methanol (Folch et al. 1957), and a 30 μl aliquot of organic solvent was applied onto the thin-layer chromatography (TLC) plate, where free cholesterol and CE were separated. The areas of cholesterol and CE were scraped off and their radioactivities were measured.

For LCAT activity, lipids were extracted from 150 μl of the incubated plasma by the same method. Free cholesterol and CE were separated by TLC. The CETP and LCAT activities were calculated using the following equations:

CETP activity (nmol CE transferred/ml plasma/hr)

$= 1/t \times [FC] \times [(dpm_{prec\text{-}CE} / dpm_{total})] \times [(dpm_{total} - 0.559 \times dpm_{HDL})/(dpm_{prec\text{-}FC} + dpm_{prec\text{-}CE})]$

LCAT activity (nmol CE esterified/ml plasma/hr)

$= 1/t \times [FC] \times dpm_{CE}/(dpm_{FC} + dpm_{CE})$

where dpm_{total} = dpm of 50 μl plasma; dpm_{HDL} = dpm of 100 μl HDL supernatant; $dpm_{prec\text{-}FC}$ = dpm of LDL+VLDL free cholesterol separated by TLC; $dpm_{prec\text{-}CE}$ = dpm of LDL+VLDL CE separated by TLC.

3.2.6 Fatty Acid Analyses

Total lipids of HDL_2, HDL_3, and LDL were extracted according to the method of Folch et al. (1957). Cholesteryl ester and triglyceride were separated by thin-layer chromatography and transmethylated with BF_3-methanol (Morrison and Smith, 1964). The fatty acid methyl esters were analyzed by gas chromatography using a Hewlett Packard 5890 Series II with Omegawax320 column (Supelco Chromatographic, Oadville, Ontario). The data were expressed as the molar percentage of total major identified fatty acids.

3.2.7 Data Analysis

All data were expressed as mean±SD. The comparison of data in subjects with normal and high plasma glucose were analyzed with *t* test. All statistical analyses were performed using the SAS program (Cary, NC). A p-value less than 0.05 was considered statistically significant.

3.3 Animal Study

3.3.1 Diets

The composition of the five diets used in this study, all of which were formulated based on the guidelines (Report) and modifications (Second report) from American Institute of Nutrition, are shown in Table 3.1. The energy content of each dietary component were determined using physiological fuel values, that is, 4 kcal/g for carbohydrate, 4 kcal/g for protein, and 9 kcal/g for fats. All diets had the same amount of protein, vitamins, minerals and cholesterol on an energy basis, but varied in fat and carbohydrate composition. AIN-93G diet (AIN) contained 15.8 energy (en)% soybean oil, while the low fat (LF) diet contained 4.5 en% safflower oil. Three high fat diets, SFA, MUFA, and PUFA, contained 40.5 en% butter, high-oleic acid safflower oil, and safflower oil, respectively. Each high fat diet also contained an additional 4.5 en% safflower oil for supplementation of essential fatty acids and the control purposes.

3.3.2 Animals

An animal protocol for this experiment was approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. Male C57BL/6 control and CETP transgenic mice, 3-8 weeks old, were obtained from Taconic (Germantown, NY). Five control and five transgenic mice initially were used in each diet group. One control mouse in the AIN group and one transgenic mouse in each of the LF and MUFA groups were sick during the experiment and were removed from the study. All animals were

allowed to adapt to the environment for one week while being fed the LF diet prior to the 5-week dietary treatment. Animals were housed individually with free access to food and water. At the end of study, mice were sacrificed after an overnight fast. Livers were removed and stored at -70 ^{o}C and blood was collected into tubes containing a final concentration of 1 mM EDTA. Plasma was obtained after centrifugation for 10 min at 4 ^{o}C.

3.3.3 Plasma Lipids and Lipoproteins analyses

Plasma total cholesterol (TC), HDL-cholesterol (HDL-C), and triglyceride (TG) were measured enzymatically with commercial kits. HDL-C was determined after precipitating apo B-containing lipoproteins from plasma with 0.2x volume of 4% (w/w) sodium phosphotungstate, 0.5 M $MgCl_2$. LDL+VLDL-cholesterol (LDL+VLDL-C) was calculated by subtracting HDL-C from TC.

3.3.4 Plasma CETP Concentration

Plasma CETP concentration was measured by sandwich ELISA using monoclonal anti-CETP, TP2 and TP20, as described in 3.2.2.

3.3.5 Statistical Analyses

All statistical analyses were performed on the SAS (Carey, NC). The comparison of data among diet groups was analyzed by using one-way ANOVA with least significant

difference (LSD) test to determine which two means were different from each other. The data from transgenic and control mice of the same diet group were analyzed by *t* test.

Ingredient	AIN		LF		SFA, MUFA, PUFA	
	wt %	energy %	wt %	energy %	wt %	energy %
Casein	20	20.3	20	20	20	20
L-Cystine	0.3		0.3		3	
Corn Starch	39.71	39.7	51	51	10.5	10.5
Dextrose	13.2	13.2	13.2	13.2	13.2	13.2
Sucrose	10	10	10	10	10	10
Cellulose	5		5		5	
Soybean oil	7	15.8	0		0	
Safflower oil	0		2	4.5	2	4.5
Exp Oil*	0		0		18	40.5
AIN-93G mineral mix	3.5		3.5		3.5	
AIN-93G vitamin mix	1		1		1	
Choline Bitartrate	0.25		0.25		0.25	
t-Butylhydroquinone	0.0014		0.0004		0.004	
Cholesterol	0.0385		0.0362		0.0459	
Protein	20.3	20.3	19.1	20.3	24.2	20.3
Carbohydrate	63.9	63.9	70.8	75.2	41.4	34.7
Fat	7	15.8	1.9	4.5	23.9	45
Cholesterol (mg/1000 Kcal)	96.5		96.5		96.5	
Kcal/g diet	4		3.8		4.8	

Table 3.1: Composition of experimental diets. *Butter in SFA, high-oleic acid safflower oil in MUFA, safflower oil in PUFA diet.

CHAPTER 4

SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY FOR PLASMA CETP

4.1 Abstract

Cholesteryl ester transfer protein (CETP) mediates the transfer of HDL cholesterol to apoB-containing lipoproteins. Its mass and activity are increased in several pro-atherogenic conditions. The objective of this study is to develop a cost- and time-effective sandwich ELISA for plasma CETP concentration. Monoclonal anti-CETP, TP20, was used as the capture antibody, while the other biotinylated monoclonal anti-CETP, TP2, was used for detection. The results were expressed in an arbitrary unit, ng biotin-TP2 bound per μl plasma. Plasma CETP concentrations, activities and their relationship were assessed in 35 type 1 diabetic children. This assay had an intra-assay CV of 8.75% and an inter-assay CV under 10%. Plasma CETP concentration of these subjects ranged from 0.36-1.89 ng biotin-TP2/μl. CETP concentration was significantly correlated with CETP activity ($r=0.51$, $P<0.01$). In conclusion, the sandwich ELISA we

have developed carried sufficient sensitivity for assaying plasma CETP concentration in human.

4.2 Introduction

Plasma cholesteryl ester transfer protein (CETP), a 476-amino-acid glycoprotein (Drayna et al. 1987), mediates the exchange of cholesteryl ester (CE) and triglyceride between high-density lipoprotein (HDL) and apolipoprotein (apo) B-containing lipoproteins (Tall 1986). It facilitates the transfer of HDL-CE, generated by the reaction of lecithin:cholesterol acyltransferase (LCAT), to apoB-containing lipoproteins. Through this reverse cholesterol transport process, plasma lipoproteins undergo continuous remodeling, and cholesterol can be redistributed to peripheral tissues for reutilization or to liver for excretion.

Because of its close relationship with cholesterol content in lipoproteins, CETP has been suggested to play an important role in cardiovascular disease. CETP activity is higher in several high-risk conditions for atherosclerosis, including obesity (Arai et al. 1994) and type 1 diabetes mellitus (Bagdade et al. 1991). Transgenic mice expressing the CETP gene showed higher incidence of atherogenesis than their wild type counterparts (Marotti et al. 1993). Its ability to decrease HDL cholesterol, which is believed to have a protective effect against atherosclerosis, also suggested that CETP may be pro-atherogenic (Agellon et al. 1991).

Various methods of quantifying cholesteryl ester transfer protein activity have been developed, using endogenous lipoproteins (Channon et al. 1990), isolated HDL and LDL (Glenn and Melton 1996), or measuring CE mass transfer (Ritter and Bagdade 1994).

However, CETP concentration has been ignored in many studies, partially because the low plasma concentration makes the measurement difficult. The published methods for assaying CETP concentration required time- and money-consuming preparations of monoclonal antibodies and purified CETP. These limitations make these assays unavailable to some laboratories. In this study, we developed a non-radioactive sandwich enzyme-linked immunosorbent assay (ELISA) using commercially available monoclonal antibodies, TP2 and TP20. It offered sufficient sensitivity for measuring CETP concentration of human plasma, and the results were expressed in an arbitrary unit, ng biotin-TP2 bound per μl plasma.

4.3 Methods

4.3.1 Subjects

The human study protocol was approved by both Columbus Children's Hospital and The Ohio State University. Thirty-five IDDM children (12 males and 23 females, ages 5-12) were recruited from Columbus Children's Hospital. Their parents or guardians signed an informed consent statement approved by both Columbus Children's Hospital Institutional Review Board for Human Studies and The Ohio State University Institutional Review Board for Human Studies. Blood samples were withdrawn by venipuncture from each subject after an overnight fast.

4.3.2 Materials

[3H]cholesterol was purchased from Amersham (Amersham, Bucks, U.K.). Mouse monoclonal anti-CETP, TP2 and TP20, were purchased from Ottawa Heart Institute (Ontario, Canada). EZ-Link Sulfo-NHS-LC-Biotinylation kit, and turbo-TMB substrate system were purchased from Pierce (Rockford, IL). Horseradish peroxidase-conjugated avidin was purchased from Sigma Chemical Co. (St. Louis, MO). 96-well polystyrene microtiter plates were purchased from Corning (Corning, NY). All other chemicals were analytical grade.

4.3.3 Buffers and Solutions

Coating buffer contained 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6. Saturation buffer contained phosphate-buffered saline (PBS), 1% (w/v) skim milk. Working buffer contained PBS, 1% (w/v) skim milk, 0.1% (v/v) Triton X-100. Washing buffer contained PBS, 0.5% (v/v) Tween 20.

4.3.4 Biotinylation of TP2

Biotinylated-TP2 was prepared using a EZ-Link biotinylation kit from Pierce, performed according to the recommendations from the manufacturer. 20 μl PBS containing sulfo-NHS-LC-biotin was added into 250 μl PBS containing 500 μg TP2. The mixture was incubated at room temperature for 30 min, then dialyzed against PBS at 4^{o}C overnight. It was adjusted to 0.1 mg/ml with PBS, separated into small aliquots, and stored at -70^{o}C until use.

4.3.5 Plasma CETP Concentration

Polystyrene 96-well microtiter plates were used as a solid phase. 200 μl TP20 in coating buffer (1ng/ μl) was added to each well, and the plates were incubated at 37^{o}C for 1.5 hr. After removing unbound TP20, 200 μl saturation buffer was added, and the plates were incubated at 37^{o}C for 1 hr. The wells were rinsed with 250 μl PBS twice before adding in triplicate 100 μl 1:7 diluted plasma from IDDM subjects and reference plasma (pooled plasma from 4 healthy donors) in working buffer. The diluted plasma was incubated at 37^{o}C for 1 hr prior to adding to the wells. 3 wells containing only working buffer served as blanks. The plates were incubated at room temperature for 3.5 hr. The wells were washed 5 times with 250 μl washing buffer and rinsed with 250 μl PBS twice. 200 μl biotin-TP2 in working buffer (0.5 ng/ μl) was added to each well. The plates were incubated at 4^{o}C overnight. The wells were washed with 250 μl washing buffer 5 times and rinsed with 250 μl PBS twice. To each well, 200 μl horseradish peroxidase-conjugated avidin (a 1:5000 dilution of Sigma stock prepared using working buffer) was added. The plates were incubated at room temperature for 2 hr. The wells were washed 5 times with 250 μl washing buffer and rinsed with 250 μl PBS twice. 150 μl Turbo-TMB substrate solution was added and the plates were incubated at room temperature for 30 min. 100 μl 1M H_2SO_4 was then added to stop the enzyme reaction. The plates were read at 450 nm with a Spectra Max 250 microtiter plate reader from Molecular Devices (Sunnyvale, CA). The absorbances of samples from IDDM subjects were compared to that of reference plasma, and expressed as 'ng biotin-TP2 bound per μl plasma' (see below).

4.3.6 Construction of Standard Curve and Calibration of Reference Plasma

The curve of absorbance of known amounts of biotin-TP2 bound directly onto the microtiter plate was constructed. To do this, 200 μl serial diluted biotin-TP2 in coating buffer (6.25, 12.5, 25, 50, 100 ng/200 μl) was added in triplicate. On the same plate, 200 μl TP20 in coating buffer (1ng/ μl) was also added to 6 other wells, and the plates were incubated at 37^{o}C for 1.5 hr. The unbound TP20 and biotin-TP2 solution was then removed, 200 μl saturation buffer was added, and the plate was incubated at 37^{o}C for 1 hr. The wells were rinsed with 250 μl PBS twice. After incubating at 37^{o}C for 1 hr, 100 μl 1:7 diluted reference plasma in working buffer was added to 6 wells coated with TP20. The wells coated with biotin-TP2 were covered with 250 μl PBS. The plate was incubated at 37^{o}C for 3.5 hr. The wells containing reference plasma were washed, incubated with biotin-TP2 overnight at 4^{o}C, and then washed, as described above. PBS was then removed from wells directly coated with biotin-TP2. 200 μl 1:5000 diluted horseradish peroxidase-conjugated avidin in working buffer was added into all wells. The plate was then incubated, washed, color-developed, and read as described above. Absorbance was plotted as a function of amount of biotin-TP2 coated directly onto the wells, using a linear regression program. The absorbance units of reference plasma, after fitting to the regression equation, were converted to the amount of biotin-TP2 bound.

4.3.6 Free Cholesterol Concentration

Plasma free cholesterol was measured according to Deacon and Dawson (1979) with slight modification. 10 μl plasma was incubated at 37^{o}C for 20 min with 1 ml

reagent containing 3 mM sodium cholate, 0.82 mM 4-aminoantipyrine, 14 mM phenol, 50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 0.17 mM Carbowax-6000, 5.85 U/L cholesterol oxidase, and 335 U/L horseradish peroxidase. The absorbances were read at 500 nm.

4.3.7 CETP Activity

Plasma CETPA was measured in triplicate according to Channon et al (1990). Briefly, 50 μl of [^{3}H]cholesterol-albumin emulsion (0.3 μCi) was added into 500 μl plasma. The mixture was incubated at 37^{o}C for 3 hr. The radioactivity of a 50 μl aliquot was measured by a liquid scintillation counter (Beckman, Palo Alto, CA). LDL+VLDL in a 300 μl aliquot was precipitated by 60 μl 4% (w/v) sodium phosphotungstate in 0.5 M $MgCl_2$. After centrifugation, the supernatant HDL was removed and the radioactivity in 100 μl aliquot was measured. The pellet was washed twice. The lipid was extracted from the pellet (Folch et al. 1957), and a 30 μl aliquot of organic solvent was applied onto the thin-layer chromatography (TLC) plate, where cholesterol and CE were separated. The areas of cholesterol and CE were scraped off and their radioactivities were measured. The CETPA was calculated using the following equation:

CETPA (nmol CE transferred/ml plasma/hr)

$= 1/t \times [FC] \times [(dpm_{prec\text{-}CE} / dpm_{total})] \times [(dpm_{total} - 0.559 \times dpm_{HDL})/(dpm_{prec\text{-}FC} + dpm_{prec\text{-}CE})]$

where dpm_{total} = dpm of 50 μl plasma; dpm_{HDL} = dpm of 100 μl HDL supernatant; $dpm_{prec\text{-}FC}$ = dpm of LDL+VLDL free cholesterol separated by TLC; $dpm_{prec\text{-}CE}$ = dpm of LDL+VLDL CE separated by TLC.

4.3.8 Data Analysis

All data were expressed as mean±SD. All correlation and regression analyses were performed using SAS (Cary, NC). A P value less than 0.05 was considered statistically significant.

4.4 Results

4.4.1 Standard Curve

The linear absorbance response range was found between 6.125-50 ng biotin-TP2, and a standard curve was constructed by least squares linear regression (Fig. 4.1). It is assumed that within this linear range, all added biotin-TP2 was completely bound. The nonlinearity observed in the 100 ng group resulted from the saturation of binding ability of the wells.

4.4.2 Dose-Response Relationship and Calibration of Reference Plasma

Fig. 4.2 showed that the linear range for this ELISA assay is between 3.125-25 μl reference plasma. The 50 μl plasma sample, which had a ratio of plasma to working buffer of 1:1, showed unexpected high absorbance. This phenomenon may result from the

interference of apoA-I in TP2-CETP binding that can not be overcome at the Triton concentration used. Therefore, 12.5 μl plasma diluted in 7-fold of working buffer was selected as the working condition for its linearity and conservation of sample. Under this condition and using the standard curve, the CETP concentration in the reference plasma was 0.8675 ng biotin-TP2/ μl. The intra-assay CV for six measurements of reference plasma was 8.75%.

4.4.3 Plasma CETP Concentration and Activity

Plasma CETP concentration, expressed in ng biotin-TP2/μl, ranged from 0.36-1.89 (1.07±0.40; mean±SD; n=31). The intra-assay CV for 31 triplicated samples were 7.27±4.22% (mean±SD). After normalization with reference plasma and expression of the values in ng biotin-TP2/μl, the typical inter-assay CV was under 10%.

The CETP activity (30.16±13.54 nmol/μl/hr; mean±SD; n=30) was significantly correlated with CETP concentration in type 1 diabetic subjects (Pearson correlation coefficient r=0.51, P<0.01) (Fig. 4.3). There was no correlation between CETP concentration and gender, race, age, and body mass index.

4.5 Discussion

We have developed a sandwich ELISA for plasma CETP concentration using two monoclonal antibodies against human CETP. It eliminated the need for iodination of antibody in radioimmunoassay (Marcel et al. 1990), and purified CETP in competitive

ELISA (Glenn and Melton 1996). The two antibodies used in this assay are relatively inexpensive. The reference plasma can be further calibrated using plasma with known CETP concentration; then the arbitrary unit used in this assay, ng biotin-TP2/μl, can be converted into an absolute one, ng CETP/μl.

The two antibodies used in this study, TP2 and TP20, recognize different epitopes of CETP and have been shown not to interfere with each other in binding the antigen (Roy et al. 1996). TP2 recognizes C-terminal and is able to inhibit CETP activity (Yen et al. 1989), while TP20 recognizes the region between residues 183-261 and lacks the ability to inhibit CETP activity (Roy et al. 1996).

Triton X-100 in working solution is crucial in this assay because it prevents the possible interference from apoA-I (Marcel et al. 1990). CETP concentration obtained in our assay did not correlate with apoA-I concentration ($r=0.11$, $P=0.54$), which suggested the absence of apoA-I interference. Skim milk is a better choice than BSA in saturation and preventing the nonspecific binding because the former produced almost zero background, while the latter produced background values similar to samples (data not shown).

It is not necessary to run a standard curve in every plate, as long as the calibrated reference plasma is used. Storage of sample for several days at 4^{o}C, freezing at -70^{o}C, and thawing for up to two times produced similar results (1.55 ± 0.22, 1.42 ± 0.15, and 1.50 ± 0.04 ng biotin-TP2/μl, respectively, for one particular sample) . The presence of reducing agents or other detergents in the sample, such as 2-mercaptoethanol and SDS,

affected the assay outcome by denaturing immobilized antibody and/or interfering with the antibody-antigen binding (data not shown).

Clark et al (1995) have reported a similar sandwich ELISA method using other commercially unavailable monoclonal anti-CETP, 2F8 and 2E7. Although that assay had a sensitivity as low as 0.5 ng/ml, the production of desirable monoclonal antibodies is a lengthy and expensive procedure. In contrast, our assay did not have such great sensitivity, but the two antibodies used in this study can be purchased at relatively low price. Furthermore, the sensitivity presented by this assay is more than adequate for measuring CETP concentration in plasma from human or CETP transgenic mice for clinical or research purposes.

It has been shown that type 1 diabetic subjects have higher CE transfer than normal people (Bagdade et al. 1991), which may be partially responsible for the increased cardiovascular complications in these patients (Dullaart et al. 1989). The greater ability of VLDL from type 1 diabetic subjects to facilitate neutral lipid exchange has been suggested to be one of the mechanisms contributing to the enhanced CE transfer in these patients (Ritter and Bagdade 1996). Other factors, including glycemic control (Ritter and Bagdade 1994) and fatty acid composition of CE acyl chain (Green and Pittman 1991), may also regulate CE transfer. In the current study, we showed that CETP concentration is significantly correlated with CETP activity ($r=0.51$, $p<0.01$) in type 1 diabetic subjects, explaining about 25% of the variance in activity. Tato et al (1995) reported a higher correlation between CETP plasma concentration and its activity ($r=0.85$, $p<0.0001$), using exogenous lipoproteins as CETP substrates. However, the endogenous activity assay we used included more physiological factors mentioned above that could affect

CETPA. Therefore, it could explain the lower, but still statistically significant, correlation between CETP concentration and activity we discovered in this study. After controlling for CETP concentration, measured by this sandwich ELISA, the factors contributing to the altered CE transfer in type 1 diabetic subjects could be further elucidated and the pharmacological/dietary interventions could be recommended.

Figure 4.1: Standard curve of ELISA. The construction of the linear regression equation shown within the figure only used data in the range of 6.25-50 ng biotin-TP2. The assay was carried out as described in Methods. The results were expressed in mean±SD of triplicated samples.

Figure 4.2: Dose-response relationship of reference plasma. 100 µl serially diluted plasma in working buffer was added to give 3.125, 6.25, 12.5, 25, and 50 µl plasma/well. The results were mean±SD of triplicated readings.

Figure 4.3: Plasma CETP concentration is significantly correlated with CETP activity in IDDM children. 27 out of 35 subjects were involved in this study. All concentration and activity values were means of triplicated samples.

CHAPTER 5

INCREASED CHOLESTERYL ESTER TRNAFER AND CHOLESTEROL ESTERIFICATION IN TYPE 1 DIABETES - RELATIONSHIPS WITH PLASMA GLUCOSE AND ARACHIDONIC ACID

5.1 Abstract

The activities of two crucial enzymes of reverse cholesterol transport, cholesterol ester transfer protein (CETP) and lecithin:cholesterol acyltransferase (LCAT), and their relationship with lipid profile, fatty acid composition of plasma lipoproteins and fasting plasma glucose were examined in 35 type 1 diabetic children. The CETP and LCAT activities were significantly lower ($p<0.05$) in subjects with normal fasting plasma glucose levels (<6.39 mmol/l) than in those with high plasma glucose levels (10.63±3.81 vs 32.18±13.94 nmol/ml/h for CETP activity; 25.52±4.53 vs 39.52±12.52 nmol/ml/h for LCAT activity, both $p<0.05$). After adjusting for the CETP concentration, C18:2ω6 in HDL_3-triglyceride, and C16:0 and C20:4ω6 in LDL-cholesteryl ester explained additional 14%, 26%, and 30%, respectively, of the variance of CETP activity. CETP

activity was positively correlated with fasting plasma glucose, CETP concentration, LCAT activity, total cholesterol, free cholesterol, LDL-C, and LDL-cholesteryl ester, while negatively correlated with cholesteryl ester to free cholesterol ratio, LDL triglyceride to protein ratio, and LDL triglyceride to cholesteryl ester ratio. LCAT activity was found to positively correlate with CETP activity, total cholesterol, free cholesterol, LDL-C, CETP concentration, and LDL-cholesteryl ester, while negatively correlate with cholesteryl ester to free cholesterol ratio. The results observed in type 1 diabetic subjects suggested that (1) accelerated LCAT and CETP activities may result in the accumulation of LDL-cholesteryl ester; (2) fasting plasma glucose is a major determinant of CETP and LCAT activities; (3) the preference of CETP for C20:4ω6 cholesteryl ester as the substrate may be responsible for increased cardiovascular complications.

5.2 Introduction

Plasma cholesteryl ester transfer protein (CETP) and lecithin:cholesterol acyltransferase (LCAT) are two of the major enzymes that contribute to the continuous remodelling process of plasma lipoproteins. The LCAT reaction is the source of the majority of cholesteryl ester (CE) in plasma (Glomset 1979), while CETP facilitates the transfer of HDL cholesterol, mainly in the form of CE which is generated by the reaction of LCAT, to apo B-containing lipoproteins (Tall 1986). Through this reverse cholesterol transport process, peripheral tissue cholesterol can be redistributed for reutilization or to liver for excretion.

Increasing CETP activity has been reported in type 1 diabetic subjects (Bagdade et al. 1991), and has been suggested to be at least partially responsible for the high incidence of macrovascular complications in these patients. Enhanced CETP activity has also been documented in type 2 diabetic (Bagdade et al. 1993, Jones et al. 1996), obese (Arai et al. 1994), and hypercholesterolemic (Bagdade et al. 1991) subjects, all of whom have high risk of cardiovascular disease. Furthermore, transgenic mice expressing the CETP gene showed higher incidence of atherogenesis than their wild type counterparts (Marotti et al. 1993). Thus, CETP may be considered as pro-atherogenic due to its ability to decrease HDL cholesterol (Agellon et al. 1991), which is generally believed to have a protective effect against atherosclerosis.

The detailed mechanism as to how CETP increases the atherogenesis in type 1 diabetic patients is still not clear. In the present study we analyzed CETP and LCAT activities using endogenous lipoproteins as substrates, and examined whether these activities correlated with the lipid profiles and fatty acid compositions of the lipoproteins and fasting plasma glucose of 35 type 1 diabetic children.

5.3 Research Design and Methods

5.3.1 Subjects

The human study protocol was approved by both Columbus Children's Hospital and The Ohio State University. 35 type 1 diabetic children (12 males and 23 females, ages 5-12) were recruited by personnel at Columbus Children's Hospital. After the nature of the procedure was explained, a parent or guardian signed an informed consent statement approved by both Columbus Children's Hospital Institutional Review Board for

Human Studies and The Ohio State University Institutional Review Board for Human Studies. Blood samples were withdrawn by venipuncture from each subject after an overnight fast.

5.3.2 Materials

[^{3}H]cholesterol was purchased from Amersham (Bucks, UK). Mouse monoclonal anti-CETP, TP2 and TP20, were purchased from the Ottawa Heart Institute (Ontario, Canada). EZ-Link Sulfo-NHS-LC-Biotinylation kit, and turbo-TMB substrate system were purchased from Pierce (Rockford, IL). Horseradish perxoidase-conjugated avidin was purchased from Sigma Chemical Co. (St. Louis, MO). 96-well polystyrene microtiter plates were purchased from Corning (Corning, NY). All other chemicals were analytical grades.

5.3.3 Fasting Blood Glucose and Glycosylated Hemoglobin

Fasting blood glucose was measured with an enzymatic colorimetric kit containing hexokinase and glucose-6-phosphate dehydrogenase (Sigma). Total glycosylated hemoglobin and glycosylated hemoglobin A1c were measured with colorimetric methods following the separation by affinity resin column (Sigma).

5.3.4 Plasma Lipids and Lipoproteins Analyses

Plasma total cholesterol and TG were measured enzymatically (Stanbio Laboratory, San Antonio, TX). HDL-C was determined after precipitating apo B-containing lipoproteins from plasma. LDL-C and VLDL-C were then calculated by the Friedewald equation (1972). Plasma free cholesterol was measured according to Deacon and Dawson (1979) with slight modification. 10 μl plasma was incubated at 37^{o}C for 20 min with 1 ml reagent containing 3 mM sodium cholate, 0.82 mM 4-aminoantipyrine, 14 mM phenol, 50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 0.17 mM Carbowax-6000, 300 U/L cholesterol oxidase, and 1000 U/L horseradish peroxidase. The absorbances were read at 500 nm. Plasma lipoproteins were isolated by a single-spin density gradient ultracentrifugation method according to Havel et al (1955) and Terpstra et al. (1981) with minor adjustment. Briefly, 2 ml fresh plasma was adjusted to 1.25 g/ml by adding KBr and sucrose, followed by overlaying with 2 ml 1.225 g/ml KBr, 4 ml 1.10 g/ml KBr, and 4 ml deionized water. After centrifugation at 200,000 g at 20^{o}C for 24 hrs, fractions of VLDL, LDL, HDL_2, and HDL_3 were removed by aspiration. LDL-FC, LDL-TG, VLDL-TG, HDL_2-C, and HDL_3-C were measured with commercial kits (Sigma). The concentration of LDL-CE was estimated by the difference between LDL-C and LDL-FC.

5.3.5 Plasma CETP Concentration, CETP and LCAT Activities

Plasma CETP concentration was measured by sandwich ELISA as described previously (Chang et al. 1999). The result was expressed as "ng biotin-TP2 bound/μl plasma" after comparing to calibrated reference plasma. Plasma CETP and LCAT activities were measured using endogenous lipoproteins according to Channon et al. (1990).

5.3.6 Fatty Acid Analyses

Total lipids of HDL_2, HDL_3, and LDL were extracted according to the method of Folch et al. (1957). Cholesteryl ester and triglyceride were separated by thin-layer chromatography and transmethylated with BF_3-methanol (Morrison and Smith 1964). The fatty acid methyl esters were analyzed by gas chromatography using a Hewlett Packard 5890 Series II with Omegawax320 column (Supelco Chromatographic, Oadville, Ontario). The data were expressed as the molar percentage of total major identified fatty acids.

5.3.7 Data Analysis

All data were expressed as mean±SD. The comparison of data in subjects with normal and high plasma glucose were analyzed with *t* test. All statistical analyses were performed using the SAS program (Cary, NC). A p-value less than 0.05 was considered statistically significant.

5.4 Results

The clinical and plasma lipid profiles of subjects with normal and high fasting plasma glucose levels are given in Table 5.1. Plasma TC and LDL-C were significantly higher in subjects with high plasma glucose than in those with normal plasma glucose levels ($p<0.05$). Subjects with normal fasting plasma glucose levels (<6.39 mmol/l, 115 mg/dL) had significantly lower CETP and LCAT activities than those with high plasma glucose levels (>6.39 mmol/l, both $p<0.05$, Table 5.2).

The fatty acid composition of cholesteryl ester and triglyceride in HDL_2, HDL_3, and LDL are shown in Table 5.3. C16:0, C18:1ω9, and C18:2ω6 were the most abundant fatty acids in all lipid fractions analyzed. C16:0 ranged from 12.32 to 31.18%, while C18:1ω9 contributed 16.85 o 36.62%, of the fatty acids. C18:2ω6 was the most dominant fatty acid in HDL_2-CE (58.86%). It also contributed 18 to 30% of the fatty acids in other lipid fractions. C20:4ω6 represented 14.42± 3.88% of the acyl groups in LDL-CE, and 10.39± 2.49%, 6.78± 2.00%, and 1.80± 0.69% of total fatty acids in HDL_3-CE, HDL_2-CE, and LDL-TG, respectively. However, C20:4ω6 represented less than 0.1% of total fatty acids in HDL_2- and HDL_3-TG.

Table 5.4 showed that CETP activity was positively correlated with the proportions of C10:0 in HDL_3-TG ($p<0.05$), C16:0 ($p<0.05$) and C20:4ω6 in LDL-CE ($p<0.01$), while negatively correlated with C18:2ω6 in HDL_3-TG ($p<0.05$). When the activity was adjusted for CETP concentration, HDL_3-TG C18:2ω6, LDL-CE C16:0, and LDL-CE C20:4ω6 contributed an additional 14%, 26%, and 30%, respectively, to the variance in CETP (Table 5.5).

The correlations among the activities of CETP, LCAT, and various lipid parameters are shown in Table 5.6. Significant positive correlations were found between CETP activity and fasting plasma glucose, CETP concentration, LCAT activity, TC, FC, LDL-C, LDL-CE, LDL protein (all $p<0.01$), and HDL_3 protein ($p<0.05$), while significant negative correlations were found between CETP activity and total CE/FC ($p<0.01$), LDL TG/protein ratio, and LDL TG/CE ratio (both $p<0.05$). LCAT activity was positively correlated with CETP activity, TC, FC, LDL-C, (all $p<0.01$), CETP concentration, HDL_3 protein, LDL protein, and LDL-CE (both $p<0.05$), while negatively correlated with total CE/FC ($p<0.01$).

5.5 Discussion

In this study, we showed that CETP and LCAT activities determined by endogenous assay were higher in type 1 diabetic children with high fasting plasma glucose than in their normal-glycemic counterparts. CETP activity has been shown to be affected by glycemic control in diabetic subjects (Ritter and Bagdade 1994), its concentration (Tato et al. 1995), fatty acyl groups on CE (Green and Pittman 1991), and the composition, structure, and concentration of its lipoprotein substrates (Eisenberg 1985, Lasuncion et al. 1990). Compared to other CETP activity assays that used pooled lipoproteins as CE donor and acceptor (Groener et al. 1986, Tall et al. 1987, Glenn and Melton 1996), the assay which we used in the present study resembled the *in vivo* situation better because it included more factors that could affect CETP activity under physiological conditions. It could also explain the relatively lower correlation ($r=0.51$, $p<0.01$) between the CETP concentration and the CETP activity shown in this study.

Our result that CETP activity is positively correlated with the fasting plasma glucose is in agreement with the report by Ritter and Bagdade (1994), who measured net CE mass transfer between endogenous lipoproteins. The effect of fasting plasma glucose on CETP activity remains statistically significant even after adjusting for the CETP concentration. It has been suggested that the increased CETP activity in type 1 diabetic patients was attributed partially to the increased activity of lipoprotein lipase (LPL) (Bagdade et al. 1994), an enzyme that is positively regulated by insulin (Schnatz and Williams 1963). It has been shown that CETP activity is positively correlated with basal plasma insulin concentration in type 2 diabetic patients (Nikkila et al. 1977). The accumulation of fatty acids on the surface of VLDL remnants after lipolysis increases the CETP activity by facilitating the binding of CETP to its substrate (Sammett and Tall 1985). CETP activity is also elevated in the postprandial state (Tall et al. 1986, Lottenberg et al. 1996) when insulin level and LPL activity are high. Conversely, CETP activity is lower in the LPL deficient subjects (Bagdade et al. 1996). Thus, the transient hyperinsulinemia resulting from insulin injection may increase CETP activity and subsequently produce the atherogenic lipid profile. This could be one of the reasons why intensive treatment in controlling blood glucose reduced the incidence of microvascular complications (Reichard et al. 1993), but had little impact on macrovascular disease in type 1 diabetic patients (Diabetes Control and Complication Trial Research Group 1993, Mann 1997). Recently, intraperitoneal injection of insulin has been adopted as a new treatment regimen to reduce the exposure of peripheral tissues to hyperinsulinemia, while offering sufficient insulin level for glycemic control (Bagdade et al. 1994). As compared

to traditional subcutaneous injection, the intraperitoneal route normalizes the accelerated LPL activity and CETP activity in type 1 diabetic patients (Bagdade et al. 1994).

We also observed that LCAT activity is higher in type 1 diabetic children with high fasting plasma glucose than in those with normal fasting plasma glucose levels. The mechanism by which insulin regulates LCAT is unclear. There are reports that LCAT activity is increased (Jones et al. 1996), decreased (Kiziltunc et al. 1997), or unchanged (Schernthaner et al. 1983, Bhatnagar et al. 1996) in diabetic subjects. Previously, a positive correlation between CETP activity and LCAT activity has been shown in normolipidemic healthy (Channon et al. 1990) and type 2 diabetic (Jones et al. 1996) subjects. The close relationship between these two enzymes indicated that an increasing CE transfer from HDL to LDL and VLDL increased cholesterol esterification in HDL. This may be due to reduction of the product inhibition of LCAT or a better HDL substrate for LCAT. Combined with the increased CE transfer from HDL to apo B-containing lipoproteins, the enhanced cholesterol esterification leads to the accumulation of CE in LDL and VLDL seen in type 1 diabetic subjects (Dullaart et al. 1989). The positive correlations between LDL-CE and CETP and LCAT activities reported in this study further support this viewpoint. The accumulation of LDL cholesterol, associated with increased CETP activity and LCAT activity in this study, has long been recognized as a risk factor for coronary heart disease (Frick et al. 1987).

Although an accelerated CETP activity is known to lower the level of HDL in transgenic mice (Agellon et al. 1991), we were unable to find this HDL-lowering effect. It is possible that this effect was offset by the simultaneously increased LCAT activity. The lack of correlation between CETP activity and HDL cholesterol in type 1 diabetic

subjects has also been reported by Dullaart et al (1989).

In this group of subjects, we discovered that C20:4ω6 and C16:0 in LDL-CE were positively correlated with CETP activity after adjusting for the CETP concentration. This phenomenon could result from (1) the specificity of CETP toward CE with these two fatty acyl groups; or (2) the preference of LCAT for these two fatty acids as substrates in synthesizing HDL-CE. LCAT also showed different preference for various fatty acids in phosphotidylcholine *in vitro* (Subbaiah and Liu 1996). Liu et al. (1995) reported that human LCAT prefers C16:0 over C20:4ω6 as substrate, even if C16:0 is in the sn-1 position while C20:4ω6 is in the sn-2 position in phosphatidylcholine. In this study, we did not observe any correlation between LCAT activity and fatty acid composition in HDL-CE in the subjects. Nonetheless, the positive correlation between the C20:4ω6 in LDL-CE and the elevated CETP activity is interesting. LDL is known to deliver arachidonic acid, in the form of cholesteryl ester, to fibroblasts (Habenicht et al. 1990), endothelial cells (Pomerantz et al. 1985), and monocytes (Salbach et al. 1992) for the synthesis of eicosanoids. This transportation is mediated by a LDL receptor-dependent mechanism (Habenicht et al. 1990). Evidence shows that arachidonic acid in the form of cholesteryl ester prefers the prostaglandin H (PGH) synthase pathway (Salbach et al. 1992) which leads to the synthesis of prostacyclin and prostaglandin E_2, both of which have potential anti-atherogenic functions (Moncada et al. 1976, Goerig et al. 1988). However, after the initial stimulation phase, arachidonic acid inhibited PGH synthase in a LDL receptor-dependent feedback mechanism (Habenicht et al. 1990). It is possible that while the PGH synthesis is inhibited, the other eicosanoid synthesis route, the 5-

lipoxygenase pathway, becomes dominant and increases the synthesis of leukotriene (LT) B_4, a chemotactic factor for adhesion of neutrophils to endothelial cells (Hoover et al. 1984), and LTC_4, a vasoconstrictor that increases vascular permeability (Dahlen et al. 1981). Thus, the accumulation of C20:4ω6 CE in LDL associated with increased CETP activity could disturb the regular eicosanoid synthesis, and be partially responsible for the increased cardiovascular complications in type 1 diabetic patients. This hypothesis is illustrated in Fig. 5.1.

In summary, we showed that in type 1 diabetic children, high fasting plasma glucose is partially responsible for the increased CETP and LCAT activities that result in the accumulation of CE in LDL. The increased LDL C20:4ω6 CE correlated with accelerated CETP activity may lead to the imbalance of eicosanoid synthesis in monocytes and/or endothelial cells, and subsequently, play a role in the development of macrovascular complications in type 1 diabetic patients.

Fig 5.1: The hypothesized mechanism in which increased CETP activity affects eicosanoid synthesis. LDLR, LDL receptor; AA, arachidonic acid; PGH_2, prostaglandin H_2; PGE_2, prostaglandin G_2; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; LTC_4, leukotriene. C_4.

Variables	Fasting plasma glucose	
	Normal	High
N	4	28
Age (years)	8.0±2.7	9.3±2.0
BMI (kg/m^2)	18±2.4	18.1±3.6
Fasting glucose (mmol/l)	4.0±1.7	15.7±4.3*
Glycosylated Hb (%)	9.1±1.4	10.1±1.5
HbA1c (%)	7.3±0.9	7.9±1.0
TC (mmol/l)	3.66±0.42	4.56±0.67*
TG (mmol/l)	0.64±0.07	0.74±0.26
HDL-C (mmol/l)	1.30±0.47	1.46±0.32
HDL_2-C (mmol/l)	0.54±0.18	0.56±0.17
HDL_3-C (mmol/l)	0.81±0.34	0.96±0.22
LDL-C (mmol/l)	2.06±0.18	2.76±0.61*
VLDL-C (mmol/l)	0.30±0.03	0.34±0.12
CETP concentration (ng biotin-TP2/μl)	1.2±0.4	1.1±0.4

Table 5.1: Clinical and plasma lipid profiles of subjects with normal (<6.39 mmol/l) and high (>6.39 mmol/l) fasting plasma glucose levels and all subjects. Data were shown in Mean±SD. *High>normal, $p<0.05$; †fasting plasma glucose data were unavailable from 3 subjects.

	Fasting plasma glucose	
	Normal (n=4)	High (n=28)
CETP activity	19.63± 3.81	32.18± 13.94*
LCAT activity	25.52± 4.53	39.52± 12.52*

Table 5.2: CETP activity and LCAT activity in subjects with normal (<6.39 mmol/l) and high (>6.39 mmol/l) fasting plasma glucose levels.Values in nmol/ml/h (mean±SD). *High>normal, $p<0.05$.

	HDL_2-CE	HDL_3-CE	LDL-CE	HDL_2-TG	HDL_3-TG	LDL-TG
C10:0	<0.1	<0.1	<0.1	<0.1	8.81±8.47	<0.1
C12:0	<0.1	<0.1	0.85±0.57	<0.1	<0.1	<0.1
C14:0	<0.1	2.00±0.58	1.52±0.74	<0.1	<0.1	1.76±1.03
C16:0	12.32±0.79	20.63±3.92	25.03±5.36	27.03±5.36	31.18±7.57	23.21±3.11
C16:1ω7	1.69±0.92	3.48±1.01	3.84±1.36	0.98±1.30	<0.1	2.78±0.92
C18:0	1.00±0.65	2.12±0.56	2.57±0.85	5.70±2.04	7.33±3.97	4.24±0.85
C18:1ω9	16.85±2.41	29.17±6.57	28.96±9.37	36.62±7.46	32.14±7.55	36.15±5.21
C18:2ω6	58.86±4.18	30.00±12.84	18.52±10.98	27.21±14.01	20.54±7.47	25.09±8.42
C20:4ω6	6.78±2.00	10.39±2.49	14.42±3.88	<0.1	<0.1	1.80±0.69

Table 5.3: Fatty acid composition of cholesteryl ester and triglyceride in HDL_2, HDL_3, and LDL, presented as the percentage of total major identified fatty acids. The data were expressed as Mean±SD.

	HDL_3-TG	LDL-CE
C10:0	0.43*	NS
C16:0	NS	0.48*
C18:2ω6	-0.38*	NS
C20:4ω6	NS	0.59†

Table 5.4: Pearson correlation coefficients between CETP activity and fatty acid compositions in different lipoproteins. *P<0.05; †p<0.01. NS, not significant.

Independent variables		Additional r^{2*}	Cumulative r^2
CETP concentration		--	0.26
CETP concentration	HDL_3-TG 18:2ω6	0.14†	0.40
CETP concentration	LDL-CE 16:0	0.26†	0.52
CETP concentration	LDL-CE 20:4ω6	0.30†	0.56

Table 5.5: Stepwise regression analysis quantifying the contribution of CETP concentration and lipoprotein fatty acid composition to the variance in CETP activity. *Additional r^2 compared to the model with CETP concentration alone. †$P<0.05$.

	CETP activity	LCAT activity
Fasting glucose	0.48*	NS
CETP concentration	0.51*	0.38†
CETP activity	--	0.59*
LCAT activity	0.59*	--
TC	0.63*	0.53*
FC	0.95*	0.62*
Total CE/FC	-0.83*	-0.69*
HDL-C	NS	NS
HDL_3 protein	0.36†	0.36†
LDL-C	0.57*	0.46*
LDL-CE	0.57*	0.44†
LDL protein	0.49*	0.43†
LDL TG/protein	-0.40†	NS
LDL TG/CE	-0.39†	NS
VLDL-C	NS	NS

Table 5.6: Pearson correlation coefficients among CETP activity, LCAT activity, and various lipoprotein parameters. *P<0.01; †p<0.05. NS, not significant.

CHAPTER 6

THE CHOLESTEROLEMIC EFFECTS OF DIETARY FATS IN CETP TRANSGENIC MICE

6.1 Abstract

To investigate the role of the CETP in cholesterolemic response to dietary fats, we analyzed plasma lipid profiles of CETP transgenic and control mice fed AIN-93G diet (AIN), a low fat diet (LF), and diets high in butter (SFA), high-oleic safflower oil (MUFA), and safflower oil (PUFA). In CETP transgenic mice, the SFA group had significantly higher plasma TC than the PUFA group, and higher LDL+VLDL-C than the MUFA group. In addition, the PUFA group had lower HDL-C than the MUFA group. In control mice, the MUFA and PUFA groups, but not the SFA group, showed significantly higher plasma TC than the LF and AIN groups. CETP transgenic mice consuming diets high in MUFA and PUFA had lower TC than control mice in the same diet groups. In the MUFA group, CETP transgenic mice had a lower LDL+VLDL-C to TC ratio, and a higher HDL-C to TC ratio than controls. In the present study, we showed that (1) CETP

may be partially responsible for the cholesterolemic response to dietary fats in humans, (2) CETP transgenic mice showed significantly decreased plasma TC levels than controls only after consuming diets high in MUFA and PUFA, and (3) CETP may contribute to the LDL-lowering effect of MUFA and PUFA through the combination of accelerated hepatic cholesterol uptake and the enhanced cholesteryl ester transfer to apo B-containing lipoproteins.

6.2 Introduction

Many studies have examined the effects of different dietary fats on plasma lipid profiles in humans (see McNamara 1987 and Katan et al. 1994 for reviews). In general, when replacing carbohydrate in the diet, saturated fatty acids, except stearic acid, raise plasma total and low density lipoprotein (LDL)-cholesterol. On the other hand, monounsaturated and polyunsaturated fatty acids decrease total and LDL-cholesterol. The levels of elevated total (Expert Panel, 1988) and LDL-cholesterol (Frick et al. 1987) have both been linked to the incidence of cardiovascular disease.

The mechanisms regulating the cholesterolemic effects of dietary fats are complicated and may include interactions among: 1) composition of lipoprotein surface or core (Shepherd et al. 1980), 2) hepatic LDL receptor activity (Daumerie et al. 1992), 3) hepatic VLDL production rate (Dietschy 1998), 4) apolipoprotein metabolism (Shepherd et al. 1978), 5) fecal sterol excretion (Grundy and Ahrens Jr. 1970), and 6) change in the activities of enzymes involved in lipid metabolism (Groener et al. 1991, Dietchy 1998).

Cholesteryl ester transfer protein (CETP), a key enzyme in the reverse cholesterol transport process and remodeling of lipoproteins, mediates the exchange between

cholesteryl ester in HDL and triglyceride in apo B-containing lipoproteins. Despite its HDL-lowering ability, the role of CETP in atherogenesis is still debated. On the one hand, CETP activity is elevated in several populations with high risk of cardiovascular disease, including diabetic, hypertriglyceridemic, and obese subjects. Transgenic mice expressing CETP also had higher incidence of atherosclerosis than wild type mice that do not carry this gene. On the other hand, the large and cholesteryl-ester rich HDL from CETP-deficient patients had less protective effect in preventing cholesterol accumulation in macrophages (Ishigami et al. 1994) and lower ability in promoting cholesterol-efflux, compared to the lipoprotein from normal subjects (Ishigami et al. 1994, Ohta et al. 1995). In addition, transgenic mice co-expressing CETP and apo CIII genes had less aortic lesions than mice expression apo CIII alone.

The objective of this present study was to examine the role of CETP in the changes of plasma lipoprotein profiles resulting from various dietary fats. The cholesterolemic effects of butter, high-oleic acid safflower oil, and safflower oil were compared among transgenic mice expressing human CETP gene and control mice.

6.3 Methods

6.3.1 Materials

Cholesterol and triglyceride concentration kits were obtained from Sigma Chemical Co. (St. Louis, MO). Sucrose, soybean oil, butter, and safflower oil were purchased from local grocery stores. High-oleic acid safflower oil was a gift from Loriva Supreme Food Inc. (Ronkonkoma, NY). All other dietary components were purchased from ICN Biochemicals, Inc. (Costa Mesa, CA).

6.3.2 Diets

The composition of the five diets used in this study, all of which were formulated based on the guidelines (Report) and modifications (Second report) from American Institute of Nutrition, are shown in Table 3.1. The energy content of each dietary component were determined using physiological fuel values, that is, 4 kcal/g for carbohydrate, 4 kcal/g for protein, and 9 kcal/g for fats. All diets had the same amount of protein, vitamins, minerals and cholesterol on an energy basis, but varied in fat and carbohydrate composition. AIN-93G diet (AIN) contained 15.8 energy (en)% soybean oil, while the low fat (LF) diet contained 4.5 en% safflower oil. Three high fat diets, SFA, MUFA, and PUFA, contained 40.5 en% butter, high-oleic acid safflower oil, and safflower oil, respectively. Each high fat diet also contained an additional 4.5 en% safflower oil for supplementation of essential fatty acids and the control purposes.

6.3.3 Animals

An animal protocol for this experiment was approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. Male C57BL/6 control and CETP transgenic mice, 3-8 weeks old, were obtained from Taconic (Germantown, NY). Five control and five transgenic mice initially were used in each diet group. One control mouse in the AIN group and one transgenic mice in each of the LF and MUFA groups were sick during the experiment and were removed from the study. All animals were allowed to adapt to the environment for one week while being fed the LF diet prior to the

5-week dietary treatment. Animals were housed individually with free access to food and water. At the end of study, mice were sacrificed after an overnight fast. Livers were removed and stored at -70 ^{o}C and blood was collected into tubes containing a final concentration of 1 mM EDTA. Plasma was obtained after centrifugation for 10 min at 4 ^{o}C.

6.3.4 Plasma Lipids and Lipoproteins Analyses

Plasma total cholesterol (TC), HDL-cholesterol (HDL-C), and triglyceride (TG) were measured enzymatically with commercial kits. HDL-C was determined after precipitating apo B-containing lipoproteins from plasma with 0.2x volume of 4% (w/w) sodium phosphotungstate, 0.5 M $MgCl_2$. LDL+VLDL-cholesterol (LDL+VLDL-C) was calculated by subtracting HDL-C from TC.

6.3.5 Plasma CETP Concentration

Plasma CETP concentration was measured by sandwich ELISA using monoclonal anti-CETP, TP2 and TP20, as described previously (Chang et al. 1999).

6.3.6 Statistical Analyses

All statistical analyses were performed on the SAS (Carey, NC). The comparison of data among diet groups was analyzed by using one-way ANOVA with least significant difference (LSD) test to determine which two means were different from each other. The data from transgenic and control mice of the same diet group were analyzed by *t* test.

6.4 Results

The plasma lipoprotein profiles of control and transgenic mice after consuming their respective experimental diets for 5 weeks are shown in Table 6.1. In control mice, the MUFA and PUFA groups showed significantly higher plasma TC than the LF and AIN groups. Compared to the LF group, on average, 86% (SFA), 64% (MUFA) and 42% (PUFA) of the increase in TC occurred in the HDL-C fraction. Control mice in the PUFA group had higher mean body weights than those in the AIN group, although body weight gains were similar among all five diet groups of control mice.

In transgenic mice, the SFA group had significantly higher plasma TC than the PUFA group, and higher LDL+VLDL-C than the MUFA group. The PUFA group had lower HDL-C than the MUFA group. The MUFA and PUFA groups weighed more than the AIN group, but the weight gain was only significantly higher in the MUFA group. The plasma CETP concentrations were not different among all five transgenic animal groups. The average daily food intake ranged from 16.39 to 20.16 kcal. The initial average body weight of each group was approximately 22 g with no significant difference among groups (data not shown).

CETP transgenic mice in MUFA and PUFA groups had lower TC than control mice in the respective diet groups. In the MUFA group, the decrease in plasma TC in transgenic mice mainly resulted from the significantly lower LDL+VLDL-C, compared to controls. The transgenic mice also had significantly lower LDL+VLDL-C to TC ratio than controls (0.21±0.09 in transgenic vs 0.36±0.09 in control, $p<0.05$). Surprisingly, the transgenic mice consuming the diet high in MUFA showed a higher HDL-C to TC ratio

than did control mice fed the same diet (0.79±0.09 in transgenic vs 0.64±0.09 in control, $p<0.05$). Transgenic mice gained less weight than controls in AIN and SFA groups (1±1 vs 4±2 in AIN, 2±1 vs 5±1 in SFA, both $p<0.05$).

6.5 Discussion

To our knowledge, this is the first study investigating the cholesterolemic effects of dietary fats in CETP transgenic mice. Mice have distinct lipid metabolism from humans, including the homogenous HDL which is the major plasma lipoprotein (LeBoeuf et al. 1983), increased hepatic lipase activity (Olivecrona et al. 1986), the lack of correlation between lipoprotein lipase activity and HDL-C concentrations (Clee et al. 1997), and the absence of CETP. However, we discovered in this study that in CETP transgenic mice, the plasma cholesterol response to dietary fats resembled that in humans. In CETP transgenic mice, the diet high in saturated fatty acids produced higher plasma TC and LDL+VLDL-C than the PUFA and MUFA diet, respectively. The lower HDL-C observed in the PUFA diet, compared to MUFA diet, was also reported in humans (Mata et al. 1992a,b). Thus, CETP may play a role in the cholesterolemic responses to dietary fats in humans. This is in agreement with the finding that transgenic mice expressing both human CETP and apo B genes had plasma lipid profiles comparable to humans (Grass et al. 1995).

In this study, we found that CETP transgenic mice that did not express human apo B had lower plasma TC than controls in the MUFA and PUFA groups. In addition, we failed to observe the HDL-lowering effect of CETP in any of the diet groups. Agellon et al. (1991) were also unable to detect a difference between CETP transgenic and control

mice in plasma TC, HDL-C, and LDL+VLDL-C in regular chow and a high fat diet without stimulation of CETP expression. However, after supplementation with Zn to induce CETP expression, these researchers were able to find significantly lower HDL-C, but not TC, in CETP transgenic mice compared to controls. In a subsequent study by the same group using more animals, a significant reduction in both HDL-C and TC were discovered in CETP transgenic mice with and without Zn supplementation (Hayek et al. 1992). Therefore, the effect of CETP on plasma TC and HDL-C seems to be subtle in mice, and can only become significant when CETP is overexpressed or when the sample sizes used in studies are large. This may be due to the fact that HDL containing mouse apo A-I is a poor substrate for human CETP, as the insertion of human apo A-I into human CETP transgenic mice markedly increases the impact of CETP on HDL-C and TC (Hayek et al. 1992). The simultaneously increased LCAT activity in CETP transgenic mice (Francone et al. 1996) could also mask the HDL-lowering effect of CETP.

Nevertheless, we observed a significant decrease in TC in CETP transgenic mice consuming diets high in MUFA and PUFA, as opposed to control mice. In the PUFA group, the decrease in TC in transgenic mice resulted from the combination of nonsignificant reductions of 22% and 38% in HDL-C and LDL+VLDL-C, respectively, while the ratios of these two lipoproteins to TC remained similar to controls. On the other hand, the reduction of TC in the MUFA group was mainly caused by a significant 53% decrease in LDL+VLDL-C, resulting in the significantly lower ratio of such lipoproteins to TC, compared to controls consuming the same diet. Although hepatic LDL receptor mRNA is down-regulated in CETP transgenic mice consuming regular chow compared to control mice (Jiang et al. 1993), the ability of MUFA and PUFA in preventing hepatic

LDL receptor suppression may overcome the increased uptake of cholesterol to the liver and maintain the high number of LDL receptors (Kurushima et al. 1995a,b). The enhanced hepatic uptake of cholesterol, resulting from the increased number of LDL receptors and the accelerated cholesterol transfer from HDL to apo B-containing lipoproteins, could partially explain the lower LDL+VLDL-C in transgenic mice, compared to controls in respective diet groups. Both MUFA and PUFA could also accelerate the receptor-mediated LDL degradation by increasing membrane fluidity (Kuo et al. 1990). However, It has been suggested that MUFA and PUFA reduced LDL-C through different mechanisms in hamsters (Kurushima et al. 1995a) and monkeys (Brousseau et al. 1993). In hamsters, PUFA was more potent in preventing the suppression of hepatic LDL receptors than MUFA, while MUFA could increase bile production by enhancing hepatic 7α-hydroxylase activity (Kurushima et al. 1995a,b). In monkeys, dietary PUFA reduced LDL-C by the combination of decreasing apo B production rate and increasing LDL fractional catabolic rate, while MUFA mainly acted through lowering apo B production rate. The more profound effect of CETP in lowering LDL+VLDL-C in the MUFA group, compared to the PUFA group, suggested that CETP may enhance the fractional catabolic rate of apo B-containing lipoproteins and/or bile secretion in the MUFA group. Furthermore, when human subjects with normal CETP activity consumed a high MUFA diet, compared to high PUFA and SFA diets, HDL_3 were more effective in promoting cellular LDL degradation when incubated with human fibroblasts *in vitro*. These HDL_3 obtained after the MUFA diet, also had higher ability to promote cellular cholesterol efflux from human fibroblasts, possibly due to their higher fluidity and the smaller sizes than those obtained after PUFA and SFA diets. Thus, HDL_3

from the MUFA diet may increase LDL receptor activity in extrahepatic tissues by stimulating cellular cholesterol efflux (Sola et al. 1993). This enhanced LDL degradation resulting from HDL_3 isolated after the MUFA diet may be one of the mechanisms in which CETP enhances LDL degradation.

Unexpectedly, transgenic mice in the MUFA group showed a higher HDL-C to TC ratio than controls. It could result from the significantly decreased LDL+VLDL fractions in transgenic mice compared to controls, while HDL fraction remained similar. In addition, as mentioned above, a diet rich in MUFA may result in HDL_3 with higher ability to promote cellular cholesterol efflux, compared to diets high in PUFA and SFA (Sola et al. 1993). This stronger effect of MUFA, combined with the similar stimulatory effect of CETP in cholesterol efflux from cells (Francone et al. 1996), may result in the higher HDL in transgenic mice than controls in the MUFA group. On the other hand, the ability of HDL in other diet groups to enhance cellular cholesterol efflux may not be potent enough to produce the higher HDL levels seen in transgenic mice in the MUFA group. The ability of CETP in decreasing TC in transgenic mice consuming diets high in MUFA and PUFA eliminated the hypercholesterolemic effect of these two fats seen in control animals. The lack of difference between transgenic and control mice in the SFA group may due to the fact that CE rich in saturated fatty acyl groups were poor substrates for the CETP reaction (Green and Pittman 1991, Morton and Parks 1996). The observation that there is no difference in plasma TG levels between transgenic and control mice in any diet group agreed with Grass et al. (1995).

In our study, MUFA and PUFA significantly increased TC in control mice, compared to other diets. The results were in agreement with the report of Cheema et al

(1997), who found MUFA and PUFA, compared to SFA, resulted in higher TC in C57BL/6J mice with and without added cholesterol. The increase in TC could result from the increase in HDL-C, as mice consuming the three high fat diets showed more elevated, but statistically insignificant, levels than those in LF and AIN groups. It is unclear why SFA failed to increase TC in control mice. Kuan and Dupont (1989) found that, after 8 weeks on diets containing 40 en% of fat with P/S ratio 0.24 and 30 en% of fat with P/S ratio 0.91, there was no difference in TC and HDL-C in C57BR/cdJ mice, a strain with similar hyperresponsiveness to diet-induced hypercholesterolemia as the C57BL/6 mice used in the present study. The mice maintained their plasma TC and HDL-C by modifying the fecal cholesterol excretion. In another study using C57BL/6J mice, a diet with 15 wt% cocoa butter also produced similar serum TC levels compared to 8 wt% corn oil after 18 weeks (Nishina et al. 1993).

Our data suggested that the expression of CETP in this strain of transgenic mice, in which the human CETP gene was attached to apo A-I promoter, was not regulated by dietary fats. CETP expression in transgenic mice is up-regulated by dietary cholesterol when the gene is accompanied by its natural flanking region, but not when this region is replaced by a metallothionein promoter (Jiang et al. 1992). The sterol regulatory element located between -370 and -138 bp of human CETP gene may be responsible for this stimulatory effect of cholesterol (Oliveira et al. 1996). However, as indicated by the study on nonhuman primates, the expression of apo A-I in intestine is regulated by neither dietary fats nor cholesterol, while the expression in liver is not affected by dietary fats when cholesterol consumption is low (Sorci-Thomas, 1989). Thus, in the low

dietary cholesterol level we used in this study, it is reasonable that CETP concentration did not differ among transgenic mice consuming various fats.

In the present study, we showed that (1) CETP transgenic mice had a cholesterolemic response to dietary fats similar to humans, (2) CETP transgenic mice showed significantly decreased plasma TC levels than controls only after consuming diets high in MUFA and PUFA, and (3) CETP may contribute to the LDL-lowering effect of MUFA and PUFA through the combination of accelerated hepatic cholesterol uptake and the enhanced cholesteryl ester transfer to apo B-containing lipoproteins.

CHAPTER 7

SUMMARY

7.1 Summary

This dissertation was composed of a human study and an animal investigation. A time- and cost-effective sandwich ELISA was also developed for measuring plasma CETP concentration. The objective of the human study was to investigate the physiological role of increased CETP and LCAT activities in type 1 diabetic children. The aim of the animal study was to examine the effect of CETP on the cholesterolemic response to dietary fats in control and transgenic mice.

The sandwich ELISA had enough sensitivity to measure plasma CETP concentration in humans and CETP transgenic mice. The commercial availability of the two monoclonal anti-CETP used in the ELISA makes this assay ideal for laboratories lacking the fund or expertise for producing monoclonal antibodies.

Thirty-five type 1 diabetic children were involved in the human study. CETP and LCAT activities were increased in subjects with high fasting plasma glucose levels (>6.39 mmol/l), compared to their counterparts with normal fasting plasma glucose. The

simultaneously accelerated cholesteryl ester transfer and cholesterol esterification resulted in elevated LDL cholesterol levels which are considered pro-atherogenic. The accumulation of arachidonic acid in LDL-CE, positively correlated with CETP activity, may disturb the eicosanoid biosynthesis, and subsequently, play a role in the development of macrovascular complications in type 1 diabetic patients.

In the animal study, CETP transgenic mice showed cholesterolemic responses to dietary fats similar to those in humans, but distinct from those in control mice. Plasma TC and LDL+VLDL-C were significantly lower in CETP transgenic mice consuming diets high in MUFA and PUFA, compared to controls consuming the same diets. A diet high in MUFA may be a good dietary intervention for subjects with elevated CETP activity because it produced anti-atherogenic lipid profiles, that is, reduced total and LDL cholesterol while maintained the level of HDL cholesterol.

7.2 Limitations and Suggestions

To increase understanding of the role of CETP in development of atherosclerosis, it would be highly desirable to compare type 1 diabetic children with control subjects of the same age. Moreover, a larger sample size should be used in the animal study to increase statistical power. In order to further elucidate the mechanisms of action of CETP in atherogenesis and the cholesterolemic response to fatty acids, the following studies are recommended: (1) to measure urinary eicosanoid metabolites in CETP transgenic mice; (2) to measure fecal sterol excretion, hepatic LDL receptor activity, and LDL production rate in CETP transgenic mice consuming different fats.

7.3 Conclusions

The following hypotheses were accepted or rejected:

1. The hypothesis that there is no correlation among CETP and LCAT activities, and plasma lipid profile in type 1 diabetic children was rejected.
   (a) CETP activity was positively correlated with fasting plasma glucose, CETP concentration, LCAT activity, total cholesterol, free cholesterol, LDL-C, and LDL-cholesteryl ester, while negatively correlated with cholesteryl ester to free cholesterol ratio, LDL triglyceride to protein ratio, and LDL triglyceride to cholesteryl ester ratio.
   (b) LCAT activity was positively correlated with CETP activity, total cholesterol, free cholesterol, LDL-C, CETP concentration, and LDL-cholesteryl ester, while negatively correlate with cholesteryl ester to free cholesterol ratio.
2. The hypothesis that there is no correlation between CETP activity and plasma CETP concentration in type 1 diabetic children was rejected.
   (a) CETP concentration was positively correlated with CETP activity.
3. The hypothesis that these is no difference in CETP and LCAT activities in type 1 diabetic children with normal and high fasting plasma glucose levels was rejected.
   (a) Type 1 diabetic children with high fasting plasma glucose levels had higher CETP and LCAT activities than their normoglycemic counterparts.
4. The hypothesis that CETP showed no specificity for any acyl groups of cholesteryl ester in lipoproteins was rejected.

(a) After adjusting for CETP concentration, CETP activity was positively correlated with C18:2ω6 in HDL_3-triglyceride, and C16:0 and C20:4ω6 in LDL-cholesteryl ester.

(b) The preference of CETP for using HDL-CE with C20:4ω6 as the substrate may affect eicosanoid biosynthesis, and subsequently, be partially responsible for the increased atherosclerosis in type 1 diabetic subjects.

5. The hypothesis that there is no difference in plasma TC, HDL-C, and LDL+VLDL-C among CETP transgenic mice consuming the AIN-93 diet, a low-fat diet, and diets rich in saturated, monounsaturated, and polyunsaturated fatty acids was rejected. Whereas, the hypothesis that there is no difference in plasma triglyceride and CETP concentrations among CETP transgenic mice consuming these diets was accepted.

(a) The SFA group had significantly higher plasma TC than the PUFA group, and higher LDL+VLDL-C than the MUFA group.

(b) The PUFA group had lower HDL-C than the MUFA group.

(c) Plasma triglyceride and CETP concentrations were similar in all groups.

6. The hypothesis that there is no difference in plasma total cholesterol, HDL-cholesterol, LDL+VLDL-cholesterol, triglyceride, and CETP concentrations between CETP transgenic and control mice consuming the same diet was rejected.

(a) CETP transgenic mice consuming diets high in MUFA and PUFA had lower TC than control mice in the same diet groups.

(b) In the MUFA group, CETP transgenic mice had decreased LDL+VLDL-C concentration, a lower LDL+VLDL-C to TC ratio, and a higher HDL-C to TC ratio than controls.

(c) There was no difference in these parameters between transgenic and control mice consuming the SFA, AIN, and LF diets.

7.4 Implications

This study indicated that increased CETP and LCAT activities may be partially responsible for the increased macrovascular complications in type 1 diabetic subjects. Thus, intraperitoneal insulin injection, which could prevent temporarily local hyperinsulinemia during injection while providing sufficient glycemic control, may be a better choice over traditional subcutaneous injection. In addition, the pharmacological treatment to inhibit CETP activity may be beneficial in these subjects. Furthermore, we showed that in the animal model with elevated CETP activity, diets rich in MUFA and PUFA could reduce plasma TC and LDL+VLDL-C, while MUFA is more potent than PUFA. Therefore, the dietary intervention with a diet high in MUFA may be able to reduce the risk of cardiovascular disease in type 1 diabetic subjects with increased CETP activity.

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APPENDIX A

CETP AND LCAT ACTIVITY ASSAY

Example data on 111997.doc and 111997.xls

Chemicals

^{3}H-cholesterol (TRK330, Amersham)

Scintillation cocktail, ScintiVerse II (SX12-4, Fisher)

TLC plates (Analtech, 19 channels, 500 micros)

All other chemicals were from Sigma or lab storage

I. Preparation of ^{3}H-cholesterol-BSA emulsion

1. Dissolve 150 mg bovine serum albumin in 3 ml Tris buffer (10 mM Tris, 150 mM NaCl; pH 7.4).
2. Add 20 uCi of stock ^{3}H-cholesterol (1uCi/ul) was added to 0.3 ml acetone, then inverse a few times.

3. Add the acetone solution to BSA-Tris buffer drop by drop. Gently shake the tube when adding the acetone solution.
4. After all acetone solution is added, gently inverse the tube. Do NOT vortex.
5. Flux the 3H-cholesterol-BSA emulsion with nitrogen until all acetone smell is gone.
6. Store the emulsion at 4 ^{o}C.

II. Incubation of plasma

1. Add 50 ul of 3H-cholesterol-BSA emulsion to 500 ul plasma in microcentrifuge tube. Gently inverse the tube. Do NOT vortex. Triplicate each sample.
2. Put the samples on ice immediately after adding the 3H-cholesterol-BSA emulsion.
3. Incubate all samples at 4 ^{o}C for 1 hour.
4. Incubate samples at 37 ^{o}C for 3 hours.

III. LCAT activity

1. After incubation, remove 150 ul of mixture was removed for LCAT activity assay.
2. Extract lipid in the mixture with chloroform/MeOH = 2:1 (v/v) three times. The first time, add 400 ul of chloroform/MeOH; and add 300 ul the last two times.
3. In each extraction, vortex or vigorously shake the tube after adding the solvent. Then incubate at room temperature for 15 minutes, before centrifuging at 6000 rpm for 10 minutes at room temperature. Collect all of the lower organic phase. The final total organic phase volume should be about 500-600 ul.
4. Analyze 50 ul of the organic phase by TLC.
5. Calculate LCAT activity as follows:

LCAT = 1/t * [FC] * (dpm-CE) / (dpm-CE + dpm-FC)

(in nmol/ml/h)

t = 3 (hours)

[FC]: free cholesterol concentration in plasma

dpm-CE: dpm of cholesteryl ester from TLC analyses

dpm-FC: dpm of free cholesterol from TLC analyses

IV. CETP activity assay

1. Add 60 ul of 4% sodium phosphotungstate in 0.5M $MgCl_2$ to 300 ul of incubated mixture (from step 4, Section II), to precipitate apo B-containing lipoprotein.
2. Incubate at room temperature for 10 minutes, then centrifuge at 6000 rpm for 10 minutes at room temperature.
3. Collect the supernantant, HDL portion.
4. Wash the pellet twice with 1 ml of 150 mM NaCl, 0.4% sodium phosphotungstate, 0.05M $MgCl_2$. In each wash, the pellet is suspended, vortexed, and then centrifuged at 6000 rpm for 10 minutes at room temperature. Discard the supernantant as completely as possible.
5. Extract the lipid in the pellet with chloroform/MeOH = 2:1 (v/v) for three times. Add 300 ul of solvent each time, and 300 ul water is also added in the first time to create the two-phase separation. The mixture was vortexed, incubated at room temperature for 15 minutes, then centrifuge at 6000 rpm for 10 minutes at room temperature.

6. Collect all of the lower organic phase was collected. The final total organic phase volume should be about 500-600 ul.
7. Analyze 50 ul of the organic phase by TLC.
8. Calculate CETP activity as follows:

CETP = 1/t * [FC] * dpm-prep-CE * (total dpm - 0.559 * dpm-HDL) / [total dpm * (dpm-prep-FC + dpm-prep-CE)]

(in nmol/ml/h)

t = 3 (hours)

[FC] = free cholesterol concentration in plasma

dpm-prep-CE: dpm of cholesteryl ester from TLC analyses

dpm-prep-FC: dpm of free cholesterol from TLC analyses

total dpm: dpm of 50 ul rection mixture (from step 4, section II)

dpm-HDL: dpm of 100 ul HDL protion (from step 3, section IV)

dilution factor 0.559: the paper said 5.59, which is WRONG. (HDL-dpm from 100 ul out of 360 (=300+60) ul HDL solution, while total dpm from 50 ul out of 550 ul incubation mixture. Convert dpm-HDL into 550 ul scale:

[total * (550/50) - HDL * (360/100) * (550/300)] / [total * (550/50)] =

(total - 0.6 * HDL) / total

V. TLC analysis

1. Use hexane: diethyl ether: acetic acid = 80: 20: 1 (v/v/v) as solvent. Usually 200 ml total solvent is required. Allow about 30 minutes for solvent to stay in the developing chamber before running TLC.
2. Use cholesterol and cholesteryl ester dissolved in chloroform/MeOH as standards.
3. Carefully load sample onto TLC plate, at about 1.5-2 cm above the bottom of the plate. Try to minimize the size of sample dots on the plate by applying samples drop by drop slowly. Also wait for the previous applied sample to dry a little bit before further loading.
4. Put the plate in developing chamber. Allow solvent front go to 5-10 cm from the top of the plate.
5. Take out the plate and dry in the hood for about 2 minutes.
6. Put plate into another chamber with crystal iodine in it. Wait until the spots are clear. Cholesterol should be about 1-2 cm above the original sample loading spot, cholesteryl ester should be in the solvent front (top of the plate).
7. Scratch off the gel on the cholesterol or cholesteryl ester spots with scraper (from Analtech), onto a weighing paper.
8. Add the gel into 20 ml scintillation vials with 5 ml scintillation cocktail in it.
9. Also add 100 of HDL, or 50 ul reaction mixture into 20 ml scintillation vials with 5 ml scintillation cocktail in it.
10. Count dpm values with scintillation counter.

VI. Free cholesterol assay

1. Prepare working reagent according to the recipe below

Chemical	Conc/liter	mg/50 ml
sodium cholate	3 mmol	101
4-aminoantipyrine	0.82 mmol	8.35
phenol	14 mmol	66
Na_2HPO_4	50 mmol	355
NaH_2PO_4	50 mmol	300
Carbowax-6000 (polyethylene glycol)	0.17 mmol	51
cholesterol oxidase	5.85 U	0.35
Peroxidase (horse radish)	335 U	1.675

2. Create stock solution by dissolving 50 mg cholesterol in 5 ml ethanol (10 mg/ml).
3. Create standards with concentration at 2, 1, 0.5, 0.25, 0.125, 0.0625 mg/ml by diluting stock solution with ethanol.
4. Add 1 ml working reagent to 10 ul standard or plasma. Triplicate each sample; duplicate or triplicate each standard.
5. Put the tube on ice immediately after adding working reagent.
6. Incubate all samples and standards in 37 C for 20 minutes.
7. Read absorbance at 500 nm.
8. Express data as nmol/ml.

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2. For free cholesterol assay: Sale et al. Anal Biochem Vol 142, pp347-350, 1984.

3. For free cholesterol assay: Deacon AC and Dawson JG. Clin Chem Vol 25, pp976-984, 1979.

4. For free cholesterol assay: Clinica Chimica Acta Vol 132, pp257, 1983.

APPENDIX B

SANDWICH ELISA FOR PLASMA CETP CONCENTRATION

Example data on 071498la.txt and 071498.xls

Chemicals

Anti-CETP TP2 and TP20 (Ottawa Heart Institute, 1053 Carling Ave, Ottawa, Ontario, Canada K1Y 4E9, Tel: (613) 761-5254, Fax: (613) 761-5281, Email: abuie@heartinst.on.ca. Attn: Anne Buie)

EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce)

Turbo-TMB substrate system (Pierce)

Horseradish perxoidase-conjugated avidin (Sigma)

96-well polystyrene microtiter plates (Corning)

Mini-Dialysis System (Pierce)

I. Biotinylation of TP2

1. Dissolve 500 ug TP2 in 250 ul PBS.
2. Add 20 ul PBS containing sulfo-NHS-LC-biotin into the TP2 solution.
3. Incubate the mixture at room temperature for 30 min, then dialyze in mini-dialysis system against PBS at 4 ^{o}C overnight.
4. Adjust the solution to 0.1 mg/ml with PBS, using extinction coefficient 1.3 at 280 nm. Separate into small aliquots, and store at –70 ^{o}C until use.

II. ELISA procedure

1. Coat 200 ul (200 ng) TP20 in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6, on ELISA plate.
2. Incubate at 37 ^{o}C for 1.5 hr.
3. Remove the buffer, block the wells 250 ul PBS-1% skim milk. Incubate at 37 ^{o}C for 1 hr.
4. Dilute plasma with PBS-1% skim milk-0.1% Triton in the ratio of 1:7. Incubate the mixture at 37 ^{o}C for 1hr.
5. Add 100 ul diluted plasma sample into wells. Wells with buffer only serve as control.
6. Incubate in room temperature for 3.5 hr on a shaker.
7. Wash wells 5 times with PBS-0.5% Tween for 10 min each.
8. Rinse wells with 250 ul PBS twice.
9. Add 200 ul of 200X diluted biotin-TP2 in PBs-1% skim milk-0.1% Triton to each well. Incubate at 4 C over night.
10. Wash wells with PBS-0.5% Tween 5 times.
11. Rinse wells with PBS twice.

12. Add 200 ul of 5000X diluted avidin-HRP in PBS-1% skim milk-0.1% Triton into each well. Incubate on a shaker at room temperature for 2 hrs.
13. Wash wells with PBS-0.5% Tween 5 times and rinced with PBS twice.
14. Add 150 ul Turbo-TMB substrate system into wells. Incubate at room temperature for 30 min.
15. Add 100 ul 1M H_2SO_4 into each well to stop the peroxidase reaction. Read the absorbance at 450 nm with a microtiter plate reader.

III. Standard Curve

The standards should be analyzed on the same plate with your samples.

1. In step 1 above, also add 200 ul of 100X, 200X, 400X, 800X, 1600X, 3200X dilution of biotin-TP2 with 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6, into wells.
2. Same method of blocking as above (PBS-1% skim milk).
3. In step 4-9 above, cover the 'standard curve' wells with PBS and do not add anything to these wells.
4. Incubate over night (remember these standard curve wells were in the same plate as your samples).
5. Follow step 10-14 above, add avidin-HRP, wash wells, and develop color the same way as samples.

APPENDIX C

FATTY ACID ANALYSIS

Data process: 051998la.txt

Data: 0519982e.xls, 0519983e.xls, 051998le.xls, 0519982t.xls, 0519983t.xls, 051998lt.xls

Chemicals

1. Choleteryl pentadecanoate, CE-C15 (Sigma)

2. Tripentadecanoic acid, TG-C15 (Sigma)

3. Boron trifluoride-methanol 14% (Sigma, free of water, as fresh as possible)

4. TLC plates (Analtech, channelled, or Whatman, high performance)

5. 2',7'-dichlorofluorescein (Sigma) (borrowed from Lydia Medeiros)

6. Hexane, chloroform, methanol, diethyl ether, acetic acid.

1. 500 ul of HDL_2, HDL_3, or LDL after ultracentrifugation was added to glass tubes.

2. To each tube add 10 ml chloroform/MeOH = 2:1, 2 ml 0.9% NaCl.

3. To each tube also add 50 ug (25 ul) cholesteryl ester-C15, 50 ug (25 ul) triglyceride-C15 as internal standards.

4. Shake vigorously and centrifuge for about 8-10 min.

5. Collect the lower organic layer using aspiration (or with pasteur pipette), and dry the organic solvent under nitrogen flux.

6. Add 300 ul chlorofrom to each tube to dissolve the lipids.

7. Apply all solvent in each tube to a high-performance TLC plate (Whatman). Apply 4 tubes to one plate with syringe. Apply each sample in a line with the length of about 3 cm. Develop the plate in hexane:diethyl ether: acetic acid = 70:30:1 (or 80:20:1).

8. Spray the plate with 2',7'-dichlorofluorescein (0.2% in ethanol) and visualize under UV light.

9. Scrape off CE and TG regions of the plate into glass tubes.

10. Add 1 ml methanol and 1 ml 1 N KOH/MeOH to each tube with silica gel in it.

11. Heat the tubes at 100 ^{o}C for 15 min for saponification.

12. Cool down the tubes by putting them in the hood and open the ventilation to maximum (or put on ice). It should take about 2-3 min.

13. Add 2 ml Boron trifluoride (BF3)-methanol to each tube. Heat the tubes at 100 ^{o}C for 15 min for transmethylation.

14. Cool down the tubes and add 2 ml 0.9% NaCl to stop the reaction and dissolve any water soluble compounds. Then add 1 ml hexane to the same tube to extract methyl esters.

15. Shake the tubes well, then collect the upper organic layer. Centrifugation usually is not neccessary.

16. Add 2 ml dd-water to hexane extract, to dissolve any water soluble compound. Collect the upper organic layer into a smaller tube with anhydrous sodium sulfate, to remove the water content in hexane.

17. Transfer the hexane extract into GC vials and analyze.

18. GC condition: Column: Omegawax 320 from Supelco (Bellefonte, PA). Head pressure 20 psi, injected volume 5 ul, injection temperature 200 ^{o}C, detection temperature 230 ^{o}C. Initial column temperature 120 ^{o}C hold 0 min, ramp 4 ^{o}C/min to 205 ^{o}C and hold for 15.8 min. Total running time is 37 min.

Notes

1. The extraction of total lipid should add 20 times chloroform/MeOH = 2:1 into samples (sample:solvent = 1:20). Also add 20% volume of saline (1ml sample, 20 ml chloroform/MeOH, 20%*(1+20) = 4.2 ml saline).
2. All extractions, including total lipids and hexane, should be shaken vigorously and well before centrifugation or separation.
3. Make sure the tubes are very clean, especially those used in final steps to collect hexane extract. Any contaminations may show up on chromatogram.
4. The teflon lining on the caps is necessary, otherwise the cap may be leaking and the plastic of the cap may show up on chromatogram also.
5. The filter paper in the TLC tank should be changed every time. Otherwise the acetic acid left on the filter paper may increase the acidity of the solvent and change the separation condition.

6. The above experiment was done in Ross Lab. The adjustments should be made regarding to the TLC plates and solvent system used.
7. The internal standards were fresh-prepared by dissolving the chemicals in chloroform/MeOH = 2:1 to the concentration of 2 mg/ml.

Reference

Morrison WR, Smith LM. (1964) Preparation of fatty acid methyl esters and dimethyl acetals from lipids with boron fluoride-methanol. J Lipd Res 5:600-8.

APPENDIX D

FREE CHOLESTEROL ASSAY

All chemicals are obtained from Sigma or from lab storage.

Example data on 112197.doc and 112197.xls

1. Prepare working reagent according to the recipe below

Chemical	Conc/liter	mg/50 ml
sodium cholate	3 mmol	101
4-aminoantipyrine	0.82 mmol	8.35
phenol	14 mmol	66
Na2HPO4	50 mmol	355
NaH2PO4	50 mmol	300
Carbowax-6000 (polyethylene glycol)	0.17 mmol	51
cholesterol oxidase	5.85 U	0.35
Peroxidase (horse radish)	335 U	1.675

2. Create stock solution by dissolving 50 mg cholesterol in 5ml ethanol (10 mg/ml).

3. Create standards with concentration at 2, 1, 0.5, 0.25, 0.125, 0.0625 mg/ml by diluting stock solution with ethanol.

4. Add 1 ml working reagent to 10 ul standard or plasma. Triplicate each sample; duplicate or triplicate each standard.
5. Put the tube on ice immediately after adding working reagent.
6. Incubate all samples and standards in 37 C for 20 minutes.
7. Read absorbance at 500 nm.
8. Express data as nmol/ml.

References

1. Sale et al. Anal Biochem Vol 142, pp347-350, 1984.
2. Deacon AC and Dawson JG. Clin Chem Vol 25, pp976-984, 1979.
3. Clinica Chimica Acta Vol 132, pp257, 1983.

APPENDIX E

CALIBRATION OF SCINTILLATION COUNTER

data: 111397.xls

1. Create 10 standards with ^{3}H-cholesterol-BSA emulsion (from 11/13/97,or see Appendix A). Add 10 ul of the emulsion (about 0.3 uCi) to each scintillation vial (20 ml, Fisher 03-337-4) with 10 ml scintillation cocktail (ScintiVerse, Fisher SX12-4).
2. ^{3}H-cholesterol was purchased from Amershan (TRK 330, 1uCi/ul).
3. Count the vials with liquid scintillation counter. The cpm of each standards should be as close as possible.
4. Count dpm of each standard from anther counter.
5. Use User #1 on the liquid scintillation counter, set H#: 3; AQC: Y; RCM: Y; 2SIGMA: 1.0 for all three channels; Data calculation: 5 (SL dpm, single label dpm); STANDARD DPM: average dpm of the standards. Then, save the program.
6. Count the 8 standards with User #1.
7. The counter will automatically calculate the quench curve and parameters.

Results

A: 4.010944

B: -0.0000916

C: -0.0000323

D: 0.0000000147

Equation: ln (%Efficiency) = A + B*(H#) + C*(H#)*(H#) + D*(H#)*(H#)*(H#)

1. Use user number #1 can get dpm value directly from the print out.
2. Dpm values can be calculated from other user programs by converting H# into %Efficiency with the equation shown above.

APPENDIX F

RECIPES FOR ANIMAL DIETS

	AIN-93G			LF			HF		

	Weight	Kcal	%Wt/ kcal	Weigh t	Kcal	%Wt/ kcal	Weigh t	Kcal	%Wt/ kcal
%Protein	20.3	20.30		19.1	20.3		24.2	20.3	
%CHO	63.9	63.93		70.7	75.2		41.4	34.7	
%FAT	7.0	15.75		1.9	4.5		23.9	45	
Total	91.2	100		91.7	100		89.5	100	
kcal/gm	3.998			3.8			4.8		
Ingredient									
Casein	200	800	5.0	200	800	5	200	800	5
L-Cystine	3	12	0.075	3	12	0.075	3	12	0.075
Corn Starch	397.1	1588.4	9.93	510	2040	12.75	105	420	2.625
Dyetrose	132	528	3.30	132	528	3.3	132	528	3.3
Sucrose	100	400	2.501	100	400	2.5	100	400	2.5
Cellulose	50	0	1.25	50	0	1.25	50	0	1.25
Soyben oil	70	630	1.75	0	0	0	0	0	0
Safflower oil	0	0	0	20	180	0.5	20	180	0.5
Exp Oil	0	0	0	0	0	0	180	1620	4.5
AIN-93G mineral mix	35	0	0.87	35	0	0.875	35	0	0.875
AIN-93G vitamin mix	10	40	0.25	10	40	0.25	10	40	0.25
Choline Bitartrate	2.5	0	0.0625	2.5	0	0.0625	2.5	0	0.0625
t-Butylhydro quinone	0.014	0	0.00035	0.004	0	0.0001	0.04	0	0.001
Cholesterol	0.385	0	0.0096	0.385	0	0.0096	0.385	0	0.0096
Total	999	3998		1063	4000		838	4000	

APPENDIX G

LIVER CHOLESTEROL ASSAY

Example data: 050799la.txt, 050799.xls

Chemicals

Triton: Sigma, electrophoresis grade

1. Prepare 35% (v/v) Triton solution by dissolving 3.5 ml Triton in 6.5 ml choloroform/MeOH=2:1.
2. Cut a small piece of liver weighed about 0.1 g.
3. Homogenize the liver in 2 ml chloroform/MeOH=2:1. Centrifuge at 8000 g at 4 C for 10 min.
4. Mix 1 ml of supernatant was mixed with 200 ul 0.9% NaCl.
5. Shake the organic-aqueous mixture, then centrifuge at 9000 rpm at room temperature for 10 min.
6. Collect the lower organic layer. Create six microcentrifuge tubes for each liver samples, 3 tubes for each free cholesterol and total cholesterol assays.

7. Add 50 ul organic layer into tubes containing 20 ul 35% (v/v) Triton in chloroform/MeOH.
8. Dry the organic solvent under air flux for 4 min, centrifuge briefly, then dried again under air flux for 3 min.
9. Use 10 ul of 1.25 g/L and 2.5 g/L free cholesterol in chloroform/MeOH as standards, containing 12.5 and 25 ug free cholesterol, respectively. Each standard tubes also contained 20 ul 35% (v/v) Triton. Create the blank tube with only 20 ul 35% Triton solution.
10. Prepare 75 ml free cholesterol reagent. Add 0.8 ml reagent into each sample and standard.
11. Mix well, incubate at room temperature for 30 min, then read absorbance at 500 nm.
12. Add cholesterol esterase into the remaining free cholesterol reagent to make total cholesterol reagent.
13. Add 0.8 ml reagent reagent into each sample and standard.
14. Mix well, incubate at room temperature for 30 min, then read absorbance at 500 nm.
15. The cholesterol and free cholesterol content was calculated as follows:

(cholesterol amount in 50 ul organic solvent in mg)*(660/50)*2 / (liver weight in gram)

because the volume of organic solvent after washed with 0.9% NaCl was about 660 ul, and 2 ml organic solvent was used in homogenization, while only 1 ml was used in the preparation procedure for the assay.

16. Prepare free cholesterol reagent as follows:

Chemical	Conc/liter	mg/50 ml
sodium cholate	3 mmol	101
4-aminoantipyrine	0.82 mmol	8.35
phenol	14 mmol	66
Na2HPO4	50 mmol	355
NaH2PO4	50 mmol	300
Carbowax-6000 (polyethylene glycol)	0.17 mmol	51
cholesterol oxidase	5.85 U	0.35
Peroxidase (horse radish)	335 U	1.675

17. For total cholesterol reagent, add cholesterol esterase into free cholesterol reagent to the fianl concentration of 500 U/L.

Reference

Carlson SE, Glodfarb S. Clinica Chimica Acta 79:575-582, 1977.

APPENDIX H

HUMAN STUDY PROTOCOL

APPENDIX I

CONSENT TO INVESTIGATIONAL TREATMENT OR PROCEDURE

APPENDIX J

ANIMAL USE PROTOCOL