REGULATION OF CHOLESTEROL ESTER TRANSFER PROTEIN BY DIETARY LIPIDS

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REGULATION OF CHOLESTEROL ESTER TRANSFER PROTEIN BY DIETARY LIPIDS

by

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Abstract

The mechanism of the regulation of cholesterol ester transfer protein by dietary fats and cholesterol was investigated using human cholesterol ester transfer protein transgenic mice fed monounsaturated fatty acid and saturated fatty acid enriched diets with or without cholesterol. Cholesterol inhibited protein activity and hepatic mRNA abundance in the monounsaturated fatty acid diet. However, cholesterol enhanced protein activity but had no effect on hepatic mRNA abundance in the saturated fatty acid diet. The molecular mechanisms of dietary lipid mediated regulation of the promoter activity of this gene were investigated using chimeric gene constructs harbouring sequential deletions of the gene promoter in SW 872 cell culture. Oleic acid and stearic acid had opposite effects indicating that the type of dietary fat alters gene regulation. There was interaction between cholesterol and fatty acids to regulate cholesterol ester transfer protein.

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List of Abbreviations

ABC	ATP-binding cassette transporter
ANOVA	analysis of variance
Apo	apolipoprotein
ATCC	American Tissue Culture Collection
ARP-1	apolipoprotein A1 regulatory protein-1
bp	base pair
BSA	bovine serum albumin
CAT	Chloramphenicol acetyl transferase
C/EBP_{α}	CCATT/ enhancer-binding protein α
CETP	cholesterol ester transfer protein
COUP-TF	chicken ovalbumin upstream transcription factor
CRE	cholesterol response element
cyp7a	cholesterol 7α hydroxylase
DMEM	Dulbecco's modified Eagle's media
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR4 (or 1, 5)	direct repeat separated by 4 (or 1, 5) nucleotides
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetic acid
Egr-1	early growth response protein-1
$FAAR/PAAR_{\delta}$	fatty acid-activated receptor
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HDL	high density lipoprotein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMG-CoA	hydroxymethylglutaryl coenzyme A
Kb	kilo base pairs
LCAT	lecithin: cholesterol acyl transferase
LDL	low density lipoprotein
LRH-1	liver receptor homologue-1
LRHBS	liver receptor homologue binding site
LXR	liver X receptor
LXRE	LXR response elements
MOPS	Morpholine propanesulfonic acid
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acids
NFR	natural flanking region
NBD-CE	nitrobenzooxadiazol-flourophor labeled cholesterol ester
25-OH	25-hydroxy
ONPG	o-nitrophenyl-β-D-galactopyranoside
PCR	polymerase chain reaction

PEA-3	polyomavirus enhancer A binding protein
PPAR	peroxisome proliferator-activated receptors
PBP	PPAR-binding protein
PBS	phosphate buffered saline
PPRE	peroxisome proliferator responsive element
PUFA	polyunsaturated fatty acids
RAR	retinoic acid receptor
RARE	retinoic acid receptor element
RNA	ribonucleic acid
RXR	retinoid X receptor
SFA	saturated fatty acids
SP-1	pregnancy specific beta-1 glycoprotein
SRC-1	steroid receptor activator-1
SRE	sterol response element
SREBP	sterol response element binding protein
TE	Tris-EDTA buffer
TG	transgenic
TLC	thin layer chromatography
VLDL	very low density lipoprotein
YY1	Ying Yang 1

Chapter 1: Introduction

1.1 Atherosclerosis and Cholesterol Ester Transfer Protein

Atherosclerosis is a leading cause of morbidity and mortality in the North American population, and plasma cholesterol levels are a major contributing factor. Death rates from coronary heart disease rise in a curvilinear fashion as a function of total plasma cholesterol (Dietschy, 1998). The increased levels of total plasma cholesterol, in particular an increase in plasma low density lipoprotein (LDL) - or very low density lipoprotein (VLDL) - cholesterol and a decrease in plasma high density lipoprotein (HDL) cholesterol, are related to an increased risk of atherosclerosis. The ratio of plasma HDL- to LDL-cholesterol is regulated by several factors, i.e. nutrients, hormones, and lipid transfer proteins such as cholesterol ester transfer protein (CETP).

CETP is a 74 kDa hydrophobic glycoprotein and a member of the lipid transfer/ lipopolysaccharide binding protein gene family (Bruce *et al.*, 1998a). It plays a major role in the reverse cholesterol transport pathway which removes cholesterol from the peripheral tissues and returns it to the liver. Cholesterol, taken up from the peripheral tissues by HDL, is esterified by lecithin: cholesterol acyl transferase (LCAT). CETP facilitates the transfer of esterified cholesterol in HDL in exchange for triglycerides in the apolipoprotein B containing lipoproteins, VLDL and LDL. The cholesterol ester in the lipoproteins is then removed from circulation by the liver via LDL-receptors.

Cholesterol is also delivered to the liver from the diet in chylomicrons. The liver is the main site of cholesterol metabolism where the fate of cholesterol is further decided. In the liver, cholesterol may be catabolized to bile acids via the rate limiting enzyme

cholesterol 7α–hydroxylase (cyp7a) and secreted from the body as bile, or cholesterol is packaged into VLDL and secreted from the liver back into circulation.

1.1.1 CETP as an atherogenic protein

CETP is a puzzling protein as it may be considered atherogenic or anti-atherogenic (Fielding *et al.*, 1996). The decrease in HDL cholesterol caused by removing cholesterol ester from HDL in exchange for triglycerides by CETP leads this protein to be considered atherogenic. CETP expression in CETP transgenic mice reduces HDL-cholesterol, an important component of the reverse cholesterol transport pathway (Agellon *et al.*, 1991). The decrease in HDL-cholesterol is accompanied by a decrease in HDL particle size and apo A-I content (Dinchuk *et al.*, 1995). CETP activity in the plasma of CETP transgenic mice increases the rate of plasma cholesterol esterification compared to control mice (Oliveira *et al.*, 1997). Genetic CETP deficiency in humans is associated with decreased CETP activity and an increase in HDL-cholesterol that is linked to a decreased risk of coronary heart disease (Moriyama *et al.*, 1998). The variation in the plasma CETP level is a major determining factor of HDL levels in hyperlipidemic men, but less so in normolipidemic men (Bruce *et al.*, 1998b). On the other hand, other reports suggest that hyper-HDL cholesterolemic Japanese men with and without a CETP deficiency have no difference in the rate of coronary heart disease (Zhong *et al.*, 1996).

In animal studies, injection of antisense oligodeoxyribonucleotides against the CETP gene in rabbits showed decreased plasma total cholesterol concentrations and

increased plasma HDL cholesterol concentrations (Sugano and Makino, 1996). The aortic cholesterol content and aortic percent lesion to total surface area also showed a significant decrease. The plasma CETP and hepatic CETP mRNA levels decreased in these rabbits indicating that repression of CETP gene expression protects against development of atherosclerosis. The CETP vaccine developed to produce anti-CETP antibodies against residues 461 to 476 of human CETP, also caused an increase in HDL-cholesterol, a decrease in LDL-cholesterol and a decrease in aortic atherosclerotic lesion area in cholesterol fed rabbits (Rittershaus *et al.*, 2000).

The CETP inhibitor JTT-705 acts to inhibit the transfer activity of CETP by forming a disulphide bond with the protein (Okamoto *et al.*, 2000). In cholesterol fed rabbits, JTT-705 increased HDL-cholesterol, decreased LDL-cholesterol and decreased atherosclerotic lesions in the aortic arch by 70% compared to control rabbits. In a human randomized phase II dose-response study, JTT-705 was found to raise HDL-cholesterol levels with minor gastrointestinal side effects (de Grooth *et al.*, 2002). The above reports direct our attention to consider that CETP is an atherogenic protein.

1.1.2 CETP as an anti-atherogenic protein

CETP is sometimes considered as an anti-atherogenic protein as it is involved in the removal of excess cholesterol from the peripheral tissues in the reverse cholesterol pathway (Fielding *et al.*, 1996). Smooth muscle cells in the intima and media of the human aorta produce CETP suggesting the arterial smooth muscle participates in removal of cholesterol esters from the arterial wall by CETP production (Ishikawa *et al.*, 2001).

The expression of CETP in CETP transgenic mice inhibits the development of atherosclerosis but only in hypertriglyceridemic mice (Hayek *et al.*, 1995). CETP expression in LCAT transgenic mice reduces atherosclerosis by restoring the functional properties of LCAT-transgenic mouse HDL-cholesterol and promoting hepatic uptake of HDL-cholesterol ester (Foger *et al.*, 1999). This supports the anti-atherogenic role of CETP in facilitating HDL-mediated reverse cholesterol transport and demonstrates the benefits of CETP expression in conditions of impaired reverse cholesterol transport.

In contrast to the report of Moriyana *et al.* (1998) in Hawaiian men of Japanese ancestry, CETP deficiency was found to be an independent risk factor for coronary heart disease despite hyper-HDL cholesterolemia (Zhong *et al.*, 1996). CETP deficiency is thought to impair the reverse cholesterol transport pathway increasing the risk of atherosclerosis despite increased HDL cholesterol levels (Zhong *et al.*, 1996). CETP deficiency impairs cholesterol ester formation in plasma while LCAT concentration and activity is normal (Oliveira *et al.*, 1997). CETP deficiency may impair the reverse cholesterol transfer pathway by secondary changes in the LCAT reaction explaining the high rates of atherosclerosis in genetic CETP deficiency.

The conflicting data regarding the role of CETP in atherosclerosis that has emerged may reflect the intricate balance between the atherogenic and anti-atherogenic properties of CETP, which can be explained by two theories. One theory is that when plasma triglycerides are high, CETP transfers cholesterol esters to VLDL, but these particles are cleared too rapidly to tilt the net flux of cholesterol towards the arterial wall (Gianturco *et al.*, 1982). Thus, under these conditions, CETP activity may inhibit

atherosclerosis by enhancing reverse cholesterol transport. On the other hand, when plasma triglycerides are normal, VLDL binds poorly to LDL receptors, hence would promote atherosclerosis (Fielding *et al.*, 1989). These reports imply the importance of CETP in maintaining cholesterol homeostasis and suggest the need to understand the regulation of this protein.

1.2 CETP gene expression

The mRNA of CETP is 1.6 Kb and is expressed in the liver, spleen, adipose tissue, heart and skeletal muscle (Jiang *et al.*, 1991). In humans, liver is the predominant source of plasma CETP but adipose tissue is also a predominant source of plasma CETP in obese subjects (Yamashita *et al.*, 2000). The CETP mRNA is expressed in mature fat cells, stromal-vascular cells and preadipocytes in humans (Radeau *et al.*, 1995). The CETP mRNA levels are higher in subcutaneous adipose tissue than visceral adipose tissue (Dusserre *et al.*, 2000). CETP gene expression in human adipose tissue is a function of cell size and the membrane cholesterol content (Radeau *et al.*, 1995). The CETP gene expression is regulated by nutrients and hormones (Bruce *et al.*, 1998a).

1.3 CETP gene structure

The CETP gene contains 16 exons and 15 introns (Agellon *et al.*, 1990). The exons occupy only 8% of the CETP gene and range in size from 32 bp to 250 bp, while the introns range in size from 87 bp to ~6000 bp. The transcription initiation site is located at –27 bp upstream of the translation initiation codon. The proximal promoter

region of the CETP gene contains a TATA box and a SP1 (pregnancy specific beta –1 glycoprotein) binding site.

The 5' regulatory region of the CETP gene is required for the regulation of CETP gene expression. The 5' regulatory region is required for the tissue-specific expression of CETP and also for the induction of CETP gene expression by saturated fat and cholesterol. This region contains binding sites for various transcription factors responsible for CETP regulation. The predominant expression of CETP in the liver and spleen of CETP transgenic mice is controlled by 5' elements between -3400 and -570 bp in the CETP promoter (Oliveira *et al.*, 1996). The -570 to – 370 bp region is also required for expression in the small intestine and other tissues, while elements in the -370 to -138 bp region contribute to adrenal expression.

The CCAAT/ enhancer-binding protein alpha (C/EBP$_\alpha$) has been found to *trans* - activate the human CETP gene promoter (Agellon *et al.*, 1992). The expression of CETP was significantly enhanced by coexpressing C/EBP$_\alpha$ in HepG2 cells. The C/EBP$_\alpha$ is expressed by mature adipocytes but is not expressed in stromal-vascular cells or preadipocytes, where only very low levels of C/EBP$_\alpha$ mRNA are detectable (Radeau *et al.*, 1998). C/EBP$_\alpha$ binds to the CETP gene promoter at ~ -250 to -200 bp with sequence specific binding.

A construct containing the –138 bp region of the promoter shows a 70-fold increase in activity compared to a construct containing only the –33 bp region (Gaudet *et al.*, 1995). The –138 to –33 bp region of the CETP gene promoter contains three tissue-specific DNA-protein binding sites, A, B, and C, whose cognate proteins synergistically

activate transcription. Site A (-26 to -57 bp) contains overlapping DNA protein recognition regions for the zinc-finger proteins Sp-1 and Egr-1 (early growth response protein-1), and the consensus binding sequence for AP-2. Site B (-59 to -87 bp) contains 2 PEA-3 (polyomavirus enhancer A binding protein) transcription factor binding motifs. Site C (-93 to -118 bp) binds members of the nuclear hormone superfamily of transcription factors including Apolipoprotein A1 Regulatory Protein-1 (ARP-1) and its homologue Ear-3/ COUP-TF (chicken ovalbumin upstream transcription factor) (Gaudet *et al.*, 1995). ARP-1 and/or Ear-3/ COUP-TF overexpression increased the transcriptional activity by binding to the –300 to –636 bp region of the CETP gene promoter but repressed the transcriptional activity when only the –300 region of the promoter was present. ARP-1 may be a transcriptional activator or repressor depending on promoter context. The -165 to -134 bp region of the CETP promoter is a retinoic acid receptor element (RARE) which binds the retinoic acid receptor (RAR) (Jeoung *et al.*, 1999).

1.4 Dietary fats, cholesterol and CETP regulation

Dietary saturated fats and cholesterol are known to alter CETP activity and gene expression in animal as well as human studies (Son *et al.*, 1986; Jiang *et al.*, 1992; Riemens *et al.*, 1999). The alterations in CETP are mainly due to an increase in the production and not due to a decrease in the catabolism of CETP (Bruce *et al.*, 1998a). The molecular mechanisms involved in cholesterol mediated regulation of CETP are beginning to be elucidated; however, the mechanism of regulation of CETP by dietary fats is unknown.

In human studies of CETP regulation by dietary fats, Lichtenstein *et al.* (2001) reported that the plasma CETP activity is highest in subjects consuming stick-margarine diet enriched in MUFA compared to margarines rich in PUFA, *trans* fatty acids or butter. In another study by van Tol *et al.* (1995), a diet enriched with the *trans* fatty acid elaidate increased CETP activity in human subjects compared to diets enriched with linoleate or stearate. The substitution of *trans* elaidic acid for *cis* oleic acid increases CETP activity in men with a correlated decrease in HDL-cholesterol (Abbey *et al.*, 1994). The National Cholesterol Education Program Step I diet (28% fat, 10% SFA) and a MUFA rich diet (38% fat, 22% MUFA) decrease plasma CETP concentrations compared to a SFA rich diet (38% fat, 20% SFA) in young, healthy, normolipidemic men (Jansen *et al.*, 2000).

Several reports are available on the regulation of CETP by dietary fats and cholesterol using animal models, however none of these reports clearly demonstrate the regulation of CETP by various types of dietary fats, and when fats are given along with cholesterol. St. Kitts vervet monkeys showed no difference in CETP activity among groups fed diets enriched in saturated (SFA), monounsaturated (MUFA), or n-6 polyunsaturated (PUFA) fatty acids (Fusegawa *et al.*, 2001). However, when the same diets were enriched with cholesterol the CETP activity increased. The positive correlations between CETP activity and VLDL- and LDL-cholesterol concentrations as well as the negative correlation between CETP activity and HDL-cholesterol concentrations were found in each diet group, with the highest strength of association in the saturated fat group.

In hamsters, feeding a 0.1% cholesterol-supplemented diet increased CETP activity (Kurushima *et al.*, 1995a). However, the addition of 5% oleic acid to the 0.1% cholesterol-supplemented diet decreased CETP activity, while the addition of 5% linoleic acid did not affect CETP activity. The addition of 5% palmitic acid to the 0.1% cholesterol-supplemented diet increased CETP activity (Kurushima *et al.*, 1995b).

Incidence of atherosclerosis increased significantly in transgenic mice expressing the simian CETP gene, when fed a diet high in saturated fat and cholesterol (Marotti *et al.*, 1993). In transgenic mice expressing human CETP, the plasma CETP activity and hepatic CETP mRNA levels increased significantly when animals were fed diets rich in saturated fats and cholesterol (Jiang *et al.*, 1992). The increase in hepatic CETP mRNA is caused by an increase in CETP transgene transcription due to the response of DNA sequences in the natural flanking region (NFR) of the CETP gene to the high cholesterol, high fat diet. Cholesterol synergistically increased the CETP activity and gene expression, when given along with saturated fats in transgenic mice. The CETP transgenic apolipoprotein E (apo E) and LDL receptor knockout mice show induction of hepatic CETP gene expression in response to a high cholesterol diet (Masucci-Magoulas *et al.*, 1996). These reports indicate that hepatic CETP gene expression is regulated by a mechanism which senses plasma cholesterol levels independent of apo E and LDL receptors and does not require the classical receptor-mediated lipoprotein uptake.

The CETP transgenic mice with the human CETP gene under the control of the apolipoprotein A-I promoter have a cholesterolemic response to dietary fats similar to that of humans (Chang *et al.*, 2001). A high fat, PUFA enriched diet lowered plasma total

cholesterol in CETP transgenic mice compared to control mice and to CETP transgenic mice fed a high fat, SFA enriched diet.

Many of these animal studies used chow based diets with the addition of cholesterol and dietary fat to examine the effect of cholesterol and dietary fat on CETP regulation. However, the composition of chow based diets is not consistent between suppliers or batches leading to variation in the nutrient content of the diet which introduces unknown factors affecting CETP regulation and cholesterol metabolism. Using a semipurified diet in feeding studies may be beneficial as it ensures the nutrient content is consistent between experiments with the only factor influencing CETP regulation and cholesterol metabolism being dietary lipids.

1.5 Transcription factors involved in cholesterol-mediated CETP gene regulation

The CETP 5' regulatory region contains binding sites for various transcription factors that regulate the CETP gene expression (Figure 1.1). Cholesterol increases CETP expression in transgenic mice expressing CETP gene under the control of its natural flanking region (Oliveira *et al.*, 1996). Cholesterol mediated stimulation of the CETP gene is suggested to be mediated via several transcription factors, i.e. sterol response element binding protein (SREBP), liver X receptor (LXR), and liver receptor homologue-1 (LRH-1) (Oliveira *et al.*, 1996; Luo *et al.*, 2000; Luo *et al.*, 2001).

A high fat, high cholesterol diet increased the expression of CETP gene when a transgene composed of the –138 to –570 bp region of the CETP gene promoter linked to the human apo A-I reporter gene was expressed in the liver of transgenic mice (Oliveira

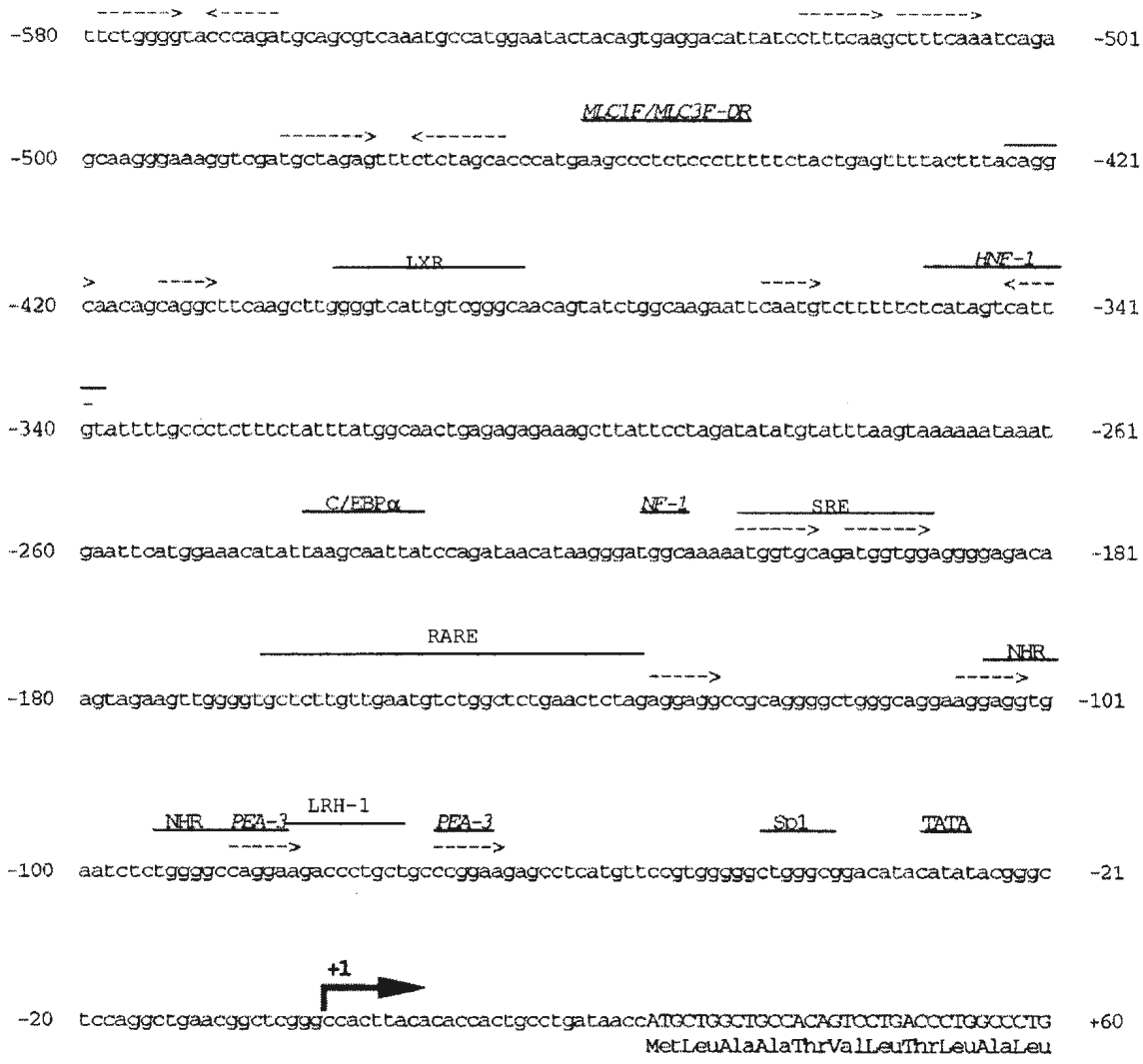


Figure 1.1: Complete nucleotide sequence between +60 and −580 bp of the human CETP promoter region. Consensus elements for known transcription factors are shown: a tandem repeat of the sterol regulatory element (SRE) of the HMG-CoA reductase gene , retinoic acid receptor element (RARE), LXR, LRH-1, the ubiquitous nuclear factor-1 (*NF-1*), C/EBP$_\alpha$, hepatocyte nuclear factor-1 (*HNF-1*), a nuclear hormone receptor (*NHR*), PEA-3, Sp1, and the muscle-specific element MLC1F. Arrows indicate direct and inverted repeats (Modified from Oliveira *et al.*, 1996).

et al., 1996). The -370 to -138 bp region contains the minimum CETP promoter element required for positive sterol response in CETP transgenic mice. This region contains a tandem repeat sterol response element (SRE) located at -211 to -187 bp identical to that which mediates sterol down regulation of the HMG-CoA (hydroxymethylglutaryl coenzyme A) reductase promoter (Chouinard *et al.*, 1998). The SRE binds SREBP-1 and Ying Yang 1 (YY1). The SREBP-1 transactivates the NFR-CETP transgene in CETP transgenic mice by binding to the SRE but this interaction is not required for positive sterol induction of CETP gene transcription by a high fat, high cholesterol diet. This suggests that the positive sterol response does not require binding of SREBP-1 and YY-1 to the CETP proximal promoter, or the induction of CETP gene expression by cholesterol requires an independent positive sterol response factor. The –361 to –138 bp region of the CETP gene promoter associated with a positive cholesterol response is designated the cholesterol response element (CRE) (Gauthier *et al.*, 1999). Deletion of the CRE results in the loss of 25-hydroxy (25-OH) cholesterol mediated regulation of CETP. SREBP-1a, -2 and YY1 *trans* activate the CRE element of the CETP promoter.

The LXR is activated by hydroxylated cholesterol (Wolf, 1999). For full activity, the LXR must form a heterodimer with the retinoid X receptor (RXR) which is activated by 9-*cis*-retinoic acid. The LXR_{α} is expressed mainly in the liver, and at lower concentrations in the kidney, intestine, spleen and adrenal glands, while LXR_{β} is expressed in most tissues. Derivatives of cholesterol *i.e.* 25-OH cholesterol and 22-OH cholesterol are potent inducers of the LXR. The sterol up regulatory response element (SURE) of the CETP gene promoter from -370 to -413 bp is required for the cholesterol

mediated induction of plasma CETP activity in CETP transgenic mice fed a high cholesterol diet (Luo *et al.*, 2000). The SURE contains a direct repeat separated by 4 nucleotides (DR4). The DR4 element 5'-GGGTCAttgtCGGGCA-3' is responsible for the sterol up regulation of the CETP promoter and is *trans* activated by $LXR_{\alpha}/RXR_{\alpha}$ and LXR_{β}/RXR_{α}. The orphan nuclear receptor LRH-1 *trans* activates the CETP promoter in a sterol-dependent manner which potentiates the effect of LXR/RXR by binding to a proximal promoter element distinct from the DR4 site (Luo *et al.*, 2001).

1.6 Fatty acids mediated gene regulation and peroxisome proliferator-activated receptors

Fatty acids regulate the expression of several genes involved in lipid metabolism via the peroxisome proliferator-activated receptors (PPARs) (Issemann *et al.*, 1990). $PPAR_{\alpha}$ is expressed in hepatocytes, cardiomyocytes, and enterocytes, while the $PPAR_{\gamma}$ is mainly expressed in adipocytes. Like LXR, the PPARs also heterodimerize with RXR and bind to the peroxisome proliferator responsive element (PPRE) located in the promoter region of the responsive genes (Jump *et al.*, 1999). Unlike LXR/RXR that binds to a DR4 site, PPREs are DR1 (direct repeat sequence separated by one nucleotide) elements consisting of the consequence sequence 5'-AGGTCAxAGGT-CA-3' (Berger *et al.*, 2002).

The PPARs are activated by peroxisome proliferators, clofibric acid, nafenopin, and WY 14643 (Jump *et al.*, 1999). Fatty acids such as caproic acid, stearic acid, oleic acid, elaidic acid, petroselinic acid, arachidonic acid and linoleic acid also activate PPAR

(Gottlicher *et al.*, 1992). Short-chain saturated fatty acids (<C10) are poor activators of $PPAR_{\alpha}$, while longer chain fatty acids (C10-C16) have weak activity (Forman *et al.*, 1997). Palmitic acid (C16:0), oleic acid (C18:1, n-9), petroselenic acid (C18:1, n-12), linolenic acid (C18:3, n-6 or n-3), linoleic acid (C18:2, n-6), and arachidonic (C20:4, n-6) acid all activate $PPAR_{\alpha}$ (Kliewer *et al.*, 1997). The PPARs also interact with the steroid receptor activator 1 (SRC-1) and PPAR-binding protein (PBP), which may act as coactivators of the PPARs (Jump *et al.*, 1999).

$PPAR_{\alpha}$ induces the expression of many genes involved in lipid metabolism including fatty acid transport protein, long chain fatty acyl-CoA synthase, enoyl-CoA hydratase /dehydrogenase multifunctional enzyme, keto-acyl-CoA thiolase, carnitine palmitoyltransferase I, HMG CoA synthase, and various acyl-CoA dehydrogenases (Berger *et al.*, 2002). PPAR activators such as fibrates are used in the treatment of hypertriglyceridemia. $PPAR_{\alpha}$ agonists also have anti-inflammatory and anti-atherosclerosis effects via $PPAR_{\alpha}$ activation inhibiting cytokine-induced vascular cell adhesion and suppressing monocyte-macrophage migration (Berger *et al.*, 2002). $PPAR_{\alpha}$ activation in macrophages induces expression of the ATP-binding cassette transporter (ABC)-A1, a cholesterol efflux pump which is also a known target of the LXR. PPARs are only one of several nuclear receptors which require the RXR as a heterodimer for DNA binding, raising the possibility that the activation of PPAR may affect other signaling networks.

The fatty acid–activated receptor (FAAR/$PPAR_{\delta}$) mediates the transcriptional effects of fatty acids in preadipocytes (Amri *et al.*, 1995). FAAR/$PPAR_{\delta}$ is highly

expressed in mouse adipose tissue, small intestine, skeletal muscle, lung, heart, and brain, and is moderately expressed in the kidney, with low expression in the liver, spleen and testis. Palmitate and 2-bromopalmitate induce the expression of FAAR/PPAR$_\delta$ mRNA in Ob1771 preadipocytes. The exposure of FAAR/PPAR$_\delta$ expressing cells to fatty acids induces the expression of mRNA for several genes. The FAAR/PPAR$_\delta$ and RXR$_\alpha$ or RXR$_\beta$ bind cooperatively to the PPRE.

1.7 SW 872 cells and CETP regulation

SW 872 is a human liposarcoma cell line that was first shown to synthesize and secrete CETP by Richardson *et al.* (1996). The SW 872 cell line has significant and reproducible levels of CETP activity. CETP secretion from SW 872 cells is 50 times greater than from HepG2 cells with the secretion of CETP into the medium increasing with time. The addition of LDL cholesterol and 25-OH cholesterol increased the secreted CETP activity and mRNA abundance in the SW 872 cells (Richardson *et al.*, 1996; Gauthier *et al.*, 1999). The addition of oleate to the growth medium in the absence of agents required for cellular differentiation caused a rapid accumulation of triglyceride-containing droplets and increased CETP activity and mRNA levels (Izem *et al.*, 2001). Transfection of SW 872 cells with the antisense oligonucleotide targeting human CETP mRNA decreases CETP secretion and cholesterol biosynthesis. SW 872 cells also express PPAR$_\alpha$ and PPAR$_\gamma$ which are important transcription factors involved in the regulation of genes of lipid metabolism (Jiang *et al.*, 2001). These findings suggest that SW 872 cells

might be an appropriate cell line to study the regulation of CETP by various fatty acids and cholesterol.

1.8 Goals

Dietary lipids are an important constituent of Western diets, and the quality of dietary fats alters the LDL-/HDL-cholesterol ratio. The CETP plays an important role in maintaining LDL-/HDL-cholesterol ratio. Saturated fats increase CETP gene expression, and the addition of cholesterol synergistically enhances the increase in CETP gene expression. Cholesterol mediated regulation of the CETP gene is via $LXR_{\alpha}/RXR_{\alpha}$, SRE and LRH-1 (Luo *et al.*, 2000; Oliveira *et al.*, 1996; Luo *et al.*, 2001). However, the mechanism for the regulation of CETP by various dietary fats is not known. It is also not known whether cholesterol, given along with various dietary fats synergistically regulates CETP.

To investigate the regulation of CETP by various dietary fats, and to understand whether cholesterol given along with various dietary fats has different effect on CETP regulation, we used transgenic mice expressing human CETP gene under the control of its natural flanking region and designated these as CETP-TG mice and age matched litter mates not expressing CETP designated as non-TG mice. Plasma CETP activity and hepatic CETP mRNA levels were used to examine the effect of dietary fats and cholesterol on CETP regulation. Cell culture studies were performed using SW 872 cells as a model to further understand the molecular mechanisms involved in dietary fat and cholesterol mediated regulation of the CETP gene. Chimeric gene constructs harbouring

serial deletions of the CETP gene promoter linked to chloramphenicol acetyl transferase as a reporter were used in transfection experiments to investigate the effects of dietary fats and cholesterol. These studies will help in understanding the regulation of CETP by dietary lipids and may prove to be beneficial in designing therapeutic strategies using diet as a therapy.

Chapter 2: Methods

2.1 Reagents

Pure fatty acids (stearic acid, 18:0; oleic acid 18:1), acetyl CoA, o-nitrophenyl-β-D-galactopyranoside (ONPG), and fatty acid free-bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Oakville, ON). Fetal bovine serum (FBS), Dulbecco's modified Eagle's media (DMEM), F12 nutrient mix, human insulin, and human transferrin were purchased from Gibco-BRL Life technologies (Burlington, ON). D-*threo*-[dichloroacetyl-^{14}C] - chloramphenicol and [4-^{14}C]-cholesterol were purchased from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec). Tristearin was purchased from ICN Biomedicals (Montreal, Quebec). Bertolli Lucca Extra Virgin Olive Oil was purchased from a local supermarket.

2.2 Animals and diets

Heterozygous transgenic mice expressing human CETP (C57BL/6J background) under the control of its native flanking region (3.4 kb NFR) were a gift from Dr. Alan Tall, Columbia University, NY (Jiang *et al.*, 1992). These mice were crossed with C57BL/6J mice obtained from Charles River Laboratories (Wilmington, MA). Mice were housed under a 12-hour light/dark cycle and fed a standard chow diet prior to the initiation of the experimental diet study. At eight weeks of age, mice were tail bled and plasma was assayed for CETP activity using the CETP Activity Assay Kit (Roar Biomedical, Inc., Columbia University, NY). Based on the presence or absence of CETP activity the mice were labeled as non-transgenic (non-TG) or transgenic (CETP-TG) mice.

Eight week old CETP-TG and non-TG mice were fed a semi-purified diet (custom made basal mix without 20% fat from ICN Biomedicals, Inc. Montreal, Quebec) containing 20 % (w/w) fat from either olive oil (enriched in 18:1, a monounsaturated fatty acid; MUFA diet), or tristearin (18:0, a saturated fatty acid enriched diet; SFA diet) in the presence or absence of 1% cholesterol, for 2 weeks. Flax seed oil (1% w/w) was added to all semipurified diets to ensure an adequate supply of essential fatty acids. The fatty acid composition of the diets was determined by gas-liquid chromatography (Table 2.1). The mice were given free access to food and water. Body weight was determined at the beginning and end of the diet study. Food was replenished every other day and food consumption was recorded. At the end of the diet study mice were sacrificed and blood was collected by cardiac puncture in tubes containing EDTA. Livers were removed, weighed and quick frozen in liquid nitrogen. Liver samples were stored at -70°C until further use.

2.3 Plasma and liver lipid analysis

Lipids were extracted from liver samples by homogenizing 50 mg of liver in 2 ml chloroform/methanol (2:1) (Folch *et al.*, 1957). The lower organic phase was dried under nitrogen, and resuspended in 100 µl of isopropanol. The plasma and liver lipids were assayed for total cholesterol and triacylglycerol concentrations using enzymatic methods (kit # 402 for total cholesterol and kit # 344 for triacylglycerol, Sigma-Aldrich Diagnostics, St. Louis, MO).

Table 2.1: Fatty acid composition of the dietary fat.[1]

Fatty acid	MUFA Diet	SFA Diet
	% w/w	
14:0	0.31	0.25
16:0	0.28	0.72
16:1	10.15	15.56
18:0	2.68	51.69
18:1	63.28	13.83
18:2 (n-6)	8.67	4.87
18:3 (n-3)	4.33	12.65
ΣSFA	3.27	52.66
ΣMUFA	73.43	29.39
ΣPUFA	13.00	17.52

[1] The fatty acid composition of the MUFA and SFA diets were determined by gas chromatography as outlined in Methods. Fatty acid composition is represented as % w/w. Abbreviations used: ΣSFA, sum of saturated fatty acids, ΣMUFA, sum of monounsaturated fatty acids, ΣPUFA, sum of polyunsaturated fatty acids.

LDL and VLDL were selectively precipitated from plasma samples by $MgCl_2$/ phosphotungstic acid precipitating reagent (kit # 352-7, Sigma-Aldrich Diagnostics, St. Louis, MO). The supernatant containing the HDL-cholesterol was assayed for cholesterol concentration using enzymatic methods (kit # 402, Sigma-Aldrich Diagnostics, St. Louis, MO). The concentration of LDL-cholesterol was calculated from the total cholesterol, HDL-cholesterol, and triacylglycerol concentrations by using the conversion formula: LDL = total cholesterol – HDL – (triacylglycerols/2.2) (Friedewald *et al.*, 1972). All assays were performed in duplicate.

2.4 CETP activity assay

The CETP activity in the plasma samples was assayed using the CETP Activity Assay kit (Roar Biomedical, Inc., Columbia University, NY) or by a previously published radioactive method with modifications (Tall *et al.*, 1987). To measure the CETP activity using the fluorometric activity kit method, 2 µl of plasma was diluted to 20 µl with sterile deionized water. Ten microlitres of the diluted plasma was combined with 3 µl of donor particle (synthetic phospholipid and cholesterol esters linked to a fluorescent label nitrobenzooxadiazol-flourophor (NBD-CE) and 3 µl of acceptor particle (VLDL) in 150 µl total volume of buffer (10 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.4). The reaction was carried out at 37°C for 3 hours. The reaction mix was transferred to a 96-well microplate and read in the Spectra Max Gemini fluorescence spectrophotometer at excitation wavelength of 465 nm and emission wavelength of 535 nm. The fluorescent cholesterol ester is present in a self-quenched state when it is contained within the core of

the donor. The CETP-mediated transfer is determined by the increase in fluorescence intensity (emission wavelength, 535 nm) as the fluorescent cholesterol ester is removed from the self-quenched donor to the acceptor. The fluorescence readings were standardized to pmole NBD-CE transferred / 3 hour by plotting a standard curve to derive the relation between fluorescence intensity and mass transfer generated by dispersing the donor in isopropanol. The unquenched fluorescence intensity of the fluorescent cholesterol ester contained within the donor particle core was determined by dispersing 5 µl of donor in 2 ml of 100% isopropanol. Serial dilutions of the dispersion were made to generate a standard curve of fluorescence intensity (excitation 465 nm/emission 535 nm) versus mass of fluorescent cholesterol ester.

For the radioisotope CETP activity assay, lipoproteins (LDL; density 1.024-1.063, and HDL; density >1.125) were isolated from human plasma (Murdoch *et al.*, 1994). The HDL fraction was radiolabelled using [4-^{14}C]-cholesterol (Tall *et al.*, 1987). Plasma samples were incubated with LDL and radiolabelled HDL for 6 hours in incubation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM diethyl-p-nitrophenylphosphate). The LDL fraction was then precipitated using heparin and $MnCl_2$ on ice, and the radioactivity in the supernatant and the precipitate was counted. The results are expressed as percent cholesterol ester transferred over 6h (% CE transfer).

2.5 CETP mRNA abundance

To detect changes in CETP mRNA abundance, total RNA from mouse livers was isolated according to standard procedures (Chomczynski *et al.*, 1987) and stored at -20°C.

The RNA concentration was determined and the integrity of the RNA was checked by running a 1% agarose gel in borate buffer. Hepatic CETP mRNA concentrations were determined by reverse transcription and *in vitro* DNA amplification. Complementary DNA was synthesized from total liver RNA (1 μg) using Superscript reverse transcriptase (Gibco BRL Life Technologies, Inc., Burlington, ON) and then used as templates for *in vitro* DNA amplification. CETP and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA sequences were amplified using specific primers (human CETP sense 5'-CGTCATCTCTAACATCATGGCCGA-3', antisense 5'-ATCTTGGGCATCTTGAG-GCAGTGGA-3'; mouse GAPDH sense 5'-GAGCCAAACGGGTCATCATC-3', antisense 5'-CATCACGCCACAGCTTTCCA-3'). No amplification products were detectable in the absence of reverse transcriptase. In preliminary experiments, the PCR (polymerase chain reaction) amplifications were carried out using different numbers of cycles, the expression levels were in the linear range using 30 cycles for both CETP and GAPDH. Thus, for all subsequent experiments 30 cycles were used to amplify CETP and GAPDH. The PCR reactions were performed in duplicate, and the products were resolved on a 1.2 % agarose gel. The representative bands were quantitated using gel documentation system. The amount of CETP mRNA was normalized to GAPDH mRNA content.

2.6 Microsome preparation

Microsomes were prepared from liver homogenates as previously described (Cheema *et al.*, 1997). In brief, 300 mg liver samples were homogenized in 3 ml of buffer I (50 mM KCl, 1 mM EDTA, 100 mM K_2PO_4 pH 7.4, 50 mM KF, 5 mM DTT,

300 mM sucrose). The homogenate was centrifuged at 9000 rpm, 4°C, for 20 minutes. The supernatant was transferred to Beckman ultracentrifuge tubes and centrifuged at 35000 rpm, 4°C, for 70 minutes in the Beckman SW 55 rotor. The microsome pellet was resuspended in 500 µl of buffer II (50 mM KCl, 1 mM EDTA, 100 mM K_2PO_4 pH 7.4, 50 mM KF, 5 mM DTT) and stored at -70°C. The total protein content of microsomes was determined using the Lowry Protein Assay (Lowry *et al.*, 1951). The free cholesterol content of the microsomes was determined using the Free Cholesterol enzymatic colorimetric method (kit # 274-47109 E, Wako Chemicals Inc., Richmond, VA).

2.7 Cholesterol 7α-hydroxylase (cyp7a) assay

Microsomal cyp7a activity was measured following the conversion of [4-^{14}C]-cholesterol to 7α-hydroxy-[4-^{14}C]-cholesterol as described previously (Agellon, 1997). Microsomes containing 100 µg of protein were incubated in the presence of 125 µl of 2x cyp7a assay buffer (200 mM K_2PO_4, pH 7.4, 2 mM EDTA, 100 mM KF), 4.5 µl of the β-cyclodextrin/ [4-^{14}C]-cholesterol cocktail (45% 2-hydroxypropyl-β-cyclodextrin, 0.9% NaCl, 0.18 mg/mL cholesterol, 34 µCi [4-^{14}C]-cholesterol) and 20 µl of 10 mg/ml NADPH solution in a total volume of 250 µl at 37°C for 15 minutes. The reaction was stopped by adding 20 µl of 5 M NaOH and the reaction products were extracted using ethyl acetate. The products were separated on TLC plates using an ethyl acetate: toluene (3:2) solvent system. The plates were exposed to a phosphorimager screen, and bands corresponding to 7α-hydroxy-[4-^{14}C]-cholesterol were quantitated. The cyp7a activity was expressed as pmol/min/mg protein.

2.8 Cloning of the human CETP gene promoter and chimeric gene construction

Chimeric gene constructs harbouring sequential deletions of the human CETP gene promoter linked to chloramphenicol acetyl transferase (CAT) as a reporter (Figure 2.1) were previously designed by Dr. S. Kaur. A 1520 bp PCR fragment containing 360 bp of the 5' regulatory region, part of exon 1, intron 1 and exon 2 were generated using the published sequence of the human CETP gene (GenBank Accession numbers U71187 & M32992). The PCR product was digested with *Hind* III and the resulting fragment containing 300 bp of the CETP gene 5' regulatory region was linked to CAT in pCAT.Basic vector (Promega) and designated 300CETP.CAT. The 150CETP.CAT was constructed by digesting the 300CETP.CAT with *Xba* I. For the longer CETP gene promoter region, the 1520 bp PCR fragment was used to screen a human chromosome 16 genomic library. An *Xba* I-*Xba* I fragment containing sequences from –3420 to –138 was obtained and cloned into 150CETP.CAT to generate 3200CETP.CAT. The 3200CETP.CAT was digested with *Kpn* I to generate 650CETP.CAT. The sequences of all cloned CETP fragments were confirmed.

Restriction mapping of 3200CETP.CAT, 650CETP.CAT, and 150CETP.CAT chimeric gene constructs was used to confirm the identity of the plasmids. The published sequences of the human CETP gene and the pCAT.Basic plasmid were used to choose the restriction enzymes. Restriction digests were performed using standard techniques.

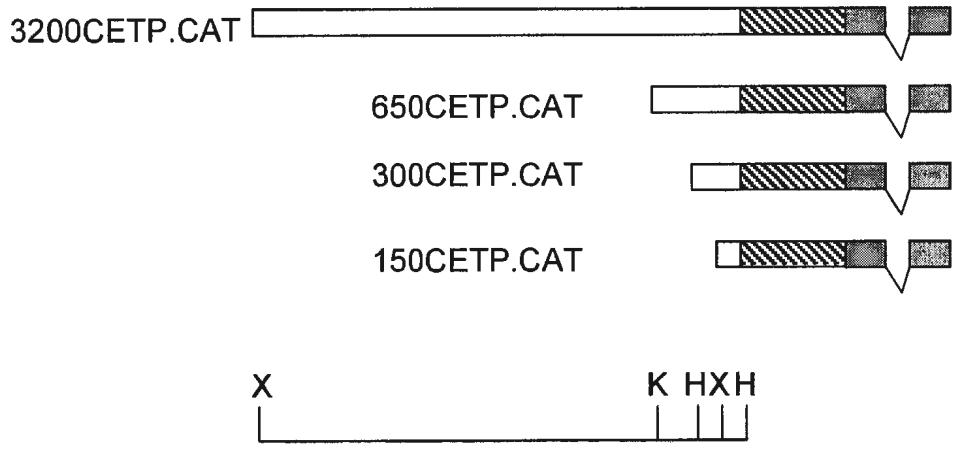


Figure 2.1: Structure of the human CETP.CAT gene chimeras. Open boxes depict the human CETP promoter; hatched boxes represent the CAT structural gene sequence; solid boxes represent the SV 40 region. X represents the digestion site for *Xba* I, K represents the digestion site for *Kpn* I, and H represents the digestion site for *Hind* III.

2.9 Preparation of competent *E. coli* DH5α cells

An overnight culture (1 ml) of DH5α cells was used to inoculate 50 ml of 2xYT medium which was incubated at 37°C to an optical density of 0.3 to 0.5 (approximately 2 hours). The culture was transferred to chilled tubes and centrifuged at 2000 rpm for 10 minutes at 4°C. The cell pellet was resuspended in 25 ml of RF1 (30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol) and incubated on ice for 30 minutes. The cells were centrifuged at 2000 rpm for 10 minutes at 4°C, resuspended in 2 ml of ice cold RF2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol) and incubated on ice for 15 minutes. The cell suspension was distributed in 100 μl portions to 1.5 ml tubes, flash frozen on dry ice, and stored at -70°C.

2.10 Introduction of DNA into *E. coli* and large scale preparation of plasmid DNA

DNA (1 μg) was added to competent DH5α cells, mixed gently and placed on ice for 30 minutes. The cells were heat shocked for 90 seconds at 42°C, returned to ice for 30 seconds and 1 ml of 2xYT media was added to the cells. The cells were incubated at 37°C for 30 minutes and selected for ampicillin resistance by plating on 2xYT media containing ampicillin. A single colony was picked to inoculate 5 ml of 2xYT media containing ampicillin, and fresh overnight culture was used to inoculate 1 litre of 2xYT media containing ampicillin. This procedure was performed for 150CETP.CAT, 650CETP.CAT, 3200CETP.CAT, C/EBP$_\alpha$, PPAR$_\alpha$, RXR, LXR$_\alpha$, pCAT.Basic, and β–galactosidase.

The standard alkaline lysis procedure was used for large scale plasmid DNA purification (Sambrook *et al.*, 1989). The DNA pellet was dissolved in 8 ml of TE buffer (5 mM Tris, pH 8.0, 1 mM EDTA) and used for CsCl density gradient centrifugation. The plasmid DNA band was collected, and ethidium bromide was removed using water-saturated n-butanol. DNA was precipitated and dissolved in low TE buffer (5 mM Tris, pH 8.0, 0.1 mM EDTA). The purity of the purified DNA plasmids was confirmed using agarose gel electrophoresis and the plasmid identity was confirmed by restriction digest as explained above in section 2.8. All DNA samples were filter sterilized and stored at -20°C.

2.11 Cell culture

SW 872 cells (human liposarcoma cells) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in a humidified atmosphere of 5% CO_2/95% air in Dulbecco's modified Eagle's media (DMEM)/ F12 (3:1), 5% FBS, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 50 μg/ml gentamycin at 37°C according to previously published methods (Richardson *et al.*, 1996). This media was designated as regular growth medium. The cells were trypsinized using 0.25% trypsin and subcultured 1:5 at 37°C, 5% CO_2.

When cells reached 70% confluence, media was removed and replaced by differentiation growth media containing DMEM/ F12 (3:1), 10 mM HEPES, 50 μg/ml gentamycin, 100 μg/ml fatty acid free BSA/ oleic acid, 1 μg/ml insulin, and 1 μg/ml transferrin for 24 h. After 24 hours, the differentiated cells were washed with phosphate

buffered saline (PBS- 0.14 M NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_20$ 1.5 mM KH_2PO_4, pH 7.4) and fresh regular growth medium (without serum) containing DMEM/ F12 (3:1), 10 mM HEPES and 50 μg/ml gentamycin was added. Cells were grown under these conditions for 24 h prior to transfection.

2.12 Oil Red O staining and lipid analysis of SW 872 cells

To investigate whether SW872 cells accumulate lipids when grown in the presence of insulin and oleic acid, the cells were grown to 70% confluency in regular growth medium. Growth media was then replaced by differentiation media or regular growth medium without FBS for 48 h. At 48 h, the cells were stained with Oil Red O (Boone *et al.*, 1986). Stained cells were viewed under a microscope for detection of lipid accumulation and photographed using a Nikon Eclipse 600 microscope with a Nikon VIII camera. For lipid extraction, the cells were grown to 70% confluency, media was aspirated and fresh medium, either regular growth medium without FBS or differentiation medium was added to the cells. The cells were harvested at various time points and lipids were extracted using chloroform/methanol (2:1) (Folch *et al.*, 1957). The lower organic phase was dried under nitrogen, and resuspended in 100 μl isopropanol. The lipids were assayed for total cholesterol and triacylglycerol concentrations using enzymatic methods (kit # 402 for total cholesterol and kit # 344 for triacylglycerol, Sigma-Aldrich Diagnostics, St. Louis, MO).

2.13 $CaPO_4$ transfection of eukaryotic cells

To investigate the effect of various fatty acids on CETP gene regulation, the CETP chimeric gene constructs (150CETP.CAT, 650CETP.CAT, and 3200CETP.CAT) were transfected into differentiated SW 872 cells. DNA-$CaPO_4$ complex was made using 0.5 ml of HBS (0.14 M NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4.7H_20$, 5.6 mM dextrose, 21 mM HEPES, pH 7.4), a total of 30 μg of DNA, and 32 μl of 2 M $CaCl_2$ (Grahem *et al.*, 1973). The total amount of DNA was kept constant by adding sonicated salmon sperm DNA. The cells were cotransfected with β-galactosidase and C/EBP_α. The β-galactosidase is used as a control for transfection efficiency and C/EBP_α is used since the coexpression of C/EBP_α is required for CETP.CAT expression in SW 872 cells (Chapter 3). The growth medium was aspirated from the cells and DNA-$CaPO_4$ complex was added to the cells and incubated for 20 minutes at room temperature. Regular growth medium containing delipidated serum (2.5% FBS) was added to the transfected cells. Fatty acids complexed to fatty acid free-BSA and 25-OH cholesterol dissolved in DMSO (dimethyl sulfoxide) were added to the transfected cells at indicated concentrations (50 μM for fatty acids and 4 μg/ml for 25-OH cholesterol). Control cells received the vehicle alone. The transfected cells were kept for 24 hours in a 5% CO_2 environment at 37 °C. Cells were washed twice with PBS, replenished with regular growth medium containing delipidated serum (5% FBS) and kept in 5% CO_2 environment at 37°C for an additional 24 hours. Cells were harvested; the cell pellet was resuspended in 175 μl of 250 mM Tris HCl, pH 8.0 and freeze/thawed six times. After centrifugation, the

supernatant was used to assay for chloramphenicol acetyl transferase activity and β-galactosidase activity.

2.14 Measurement of β-galactosidase activity

Transfected cell extract and 2x β-galactosidase assay buffer (200 mM sodium phosphate, pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 g/ml ONPG) were mixed in equal ratio and incubated at 37°C for 1 h. The reaction was stopped with 1 M sodium carbonate and the absorbance was read at 420 nm using the Spectra Max 190 plate reader.

2.15 Measurement of chloramphenicol acetyl transferase (CAT) activity

The cell extract from transfected cells was heated to 65°C for 10 minutes to inactivate endogenous deacetylase activity, cooled on ice, and mixed with a cocktail containing 2 µl of D-*threo*-[dichloroacetyl-^{14}C]- chloramphenicol and 113 µl of 250 mM Tris HCl (pH 8.0). The final volume was adjusted to 160 µl with 250 mM Tris HCl, pH 8.0. The reaction was initiated by adding 20 µl of freshly prepared acetyl CoA (3.5 mg/ml) and incubated at 37°C for 1 hour. The reaction products were extracted using ethyl acetate and separated on TLC plates using chloroform: methanol (95:5) solvent system. The TLC plates were exposed to a phosphorimager screen and bands of mono- and di-acetylated ^{14}C-chloramphenicol were quantitated. The CAT activity was normalized to β-galactosidase activity and the control samples in each experiment were assigned a value of one.

2.16 Statistical analysis

All assays were performed in duplicate. The CAT and β–galactosidase activity data from the cell transfection experiments were analyzed using Student's *t* test. The data from the mice experiments were analyzed using ANOVA. The differences between groups were considered significant if $p<0.05$ (Steele and Torie, 1980). Values without a common superscript are significantly different.

Chapter 3: Results

3.1 Effect of a high fat diet enriched in monounsaturated fatty acids (MUFA) on cholesterol metabolism

3.1.1 Body weight and liver weight of mice fed MUFA diet

Body weights were recorded to ensure that mice were eating the experimental diets. The CETP-TG and non-TG mice fed MUFA, with or without dietary cholesterol showed no significant differences in body weight over a two week feeding period (Table 3.1). There were no significant differences in liver weights in CETP-TG and non-TG mice fed MUFA diet with or without dietary cholesterol.

3.1.2 Plasma lipid levels

Changes in plasma lipid levels of CETP-TG and non-TG mice fed a diet enriched in MUFA with or without cholesterol are shown in Table 3.2. Total plasma cholesterol concentrations of CETP-TG mice fed MUFA diet were significantly lower compared to non-TG mice fed the MUFA diet (21% decrease, p=0.036). However, when cholesterol was added to the MUFA diet, there was no significant difference in the total plasma cholesterol concentrations of CETP-TG or non-TG mice. The LDL-cholesterol concentrations were not different between CETP-TG and non-TG mice fed a diet enriched in MUFA. The addition of cholesterol to the MUFA diet caused 1.30 fold (p=0.02) and 1.28 fold increase (p=0.009) in LDL-cholesterol concentrations in CETP-TG and non-TG mice respectively. The CETP-TG mice showed a significant decrease in HDL-cholesterol concentrations compared to the non-TG mice (p=0.019). Addition of cholesterol to the MUFA diet had no significant effect on HDL-cholesterol

Table 3.1: Body weight and liver weight of CETP transgenic (CETP-TG) and non transgenic (NON-TG) mice fed MUFA diet in the presence or absence of 1% cholesterol.[1]

Group	Diet	n	Original body weight (g)	Final body weight (g)	Liver weight (g)
CETP-TG	MUFA	4	21.30±1.59	23.10±1.49	1.16±0.14
	MUFA + cholesterol	8	21.84±2.71	23.81±2.85	1.18±0.21
NON-TG	MUFA	8	23.30±1.96	25.70±2.72	1.25±0.23
	MUFA + cholesterol	5	24.30±2.16	27.72±3.69	1.52±0.36

[1] Mice were weighed at the beginning and conclusion of the experiment. The livers were collected and weighed at the conclusion of the experiment. Values shown are mean ± standard deviation.

Table 3.2: Plasma lipid concentrations of CETP transgenic (CETP-TG) and non transgenic (NON-TG) mice fed a diet enriched in MUFA.[1]

Group	Diet	n	Total Cholesterol (mmol/L)	LDL-Cholesterol (mmol/L)	HDL-Cholesterol (mmol/L)	Total Triacylglycerol (mmol/L)
CETP-TG	MUFA	4	1.75 ± 0.25^{b}	0.79 ± 0.09^{bc}	0.79 ± 0.13^{b}	0.20 ± 0.04^{b}
	MUFA + cholesterol	8	1.91 ± 0.33^{ab}	1.03 ± 0.12^{a}	0.74 ± 0.15^{b}	0.24 ± 0.06^{ab}
NON-TG	MUFA	8	2.21 ± 0.27^{a}	0.71 ± 0.08^{c}	1.34 ± 0.37^{a}	0.29 ± 0.07^{ab}
	MUFA + cholesterol	5	2.35 ± 0.64^{ab}	0.91 ± 0.08^{ab}	1.85 ± 0.81^{a}	0.30 ± 0.03^{a}

[1] Values shown are mean ± standard deviation. Values without a common superscript are significantly different. ($p < 0.05$).

concentrations in CETP-TG or non-TG mice. Total plasma triacylglycerol concentrations were not significantly different between the CETP-TG and non-TG mice fed a diet enriched in MUFA. Addition of cholesterol to the MUFA diet had no significant effect on total plasma triacylglycerol concentrations in both CETP-TG and non-TG mice.

3.1.3 Hepatic lipid levels

Changes in hepatic lipid levels of CETP-TG and non-TG mice fed a high fat diet enriched in MUFA with or without cholesterol are shown in Table 3.3. There was no significant difference in hepatic total cholesterol concentrations between CETP-TG and non-TG mice. The addition of cholesterol to the MUFA diet caused a significant increase in hepatic total cholesterol concentrations for both CETP-TG and non-TG mice (2.25 fold, p=0.006 and 2.13 fold, p=0.00001 increase respectively). Hepatic total triacylglycerol concentrations of CETP-TG were 1.53 fold higher (p=0.005) than non-TG mice when fed a diet enriched in MUFA. The addition of cholesterol to the MUFA diet caused a significant increase in hepatic total triacylglycerol concentrations in both CETP-TG and non-TG mice (2.31 fold, p=0.007 and 1.56 fold, p=0.01 increase respectively).

3.1.4 Plasma CETP activity and hepatic CETP mRNA levels

The plasma CETP activity and hepatic CETP mRNA levels were measured for transgenic mice expressing CETP. Non-TG mice had undetectable CETP activity and mRNA levels. Addition of cholesterol to the MUFA diet caused significant inhibition of

Table 3.3: Hepatic lipid levels of CETP transgenic (CETP-TG) and non transgenic (NON-TG) mice fed a diet enriched in MUFA.[1]

Group	Diet	n	Total Cholesterol (mmol/mg liver)	Total Triacylglycerol (mmol/mg liver)
CETP-TG	MUFA	4	7.82 ± 1.67^{b}	61.01 ± 4.12^{bc}
	MUFA + cholesterol	8	17.59 ± 4.34^{a}	141.15 ± 24.21^{a}
NON-TG	MUFA	8	6.14 ± 1.05^{b}	39.80 ± 4.53^{d}
	MUFA + cholesterol	5	13.10 ± 1.31^{a}	61.97 ± 11.24^{b}

[1] Values shown are mean ± standard deviation. Values without a common superscript are significantly different. ($p < 0.05$).

CETP activity (56% of mice fed the MUFA diet (p=0.04) (Figure 3.1). There is a parallel reduction in hepatic CETP mRNA expression in mice fed a MUFA diet with added cholesterol (Figure 3.2).

3.1.5 Cholesterol 7α hydroxylase (cyp7a) activity

To investigate whether dietary cholesterol influences the breakdown of cholesterol in liver, the activity of cyp7a was measured. The cyp7a activity was 2.1 fold higher (p=0.0007) in CETP-TG mice compared to non-TG mice when fed a diet enriched in MUFA (Figure 3.3). The addition of cholesterol to the MUFA diet increased cyp7a activity in both non-TG and CETP-TG mice; however, the fold increase was greater for non-TG mice compared to CETP-TG mice (3.2 fold, p=0.003 increase vs. 2.4 fold, p=0.0005 increase).

3.1.6 Summary of findings when mice were fed a diet rich in MUFA

The expression of CETP gene decreases the plasma HDL-cholesterol and increases hepatic triacylglycerol and cyp7a activity. The addition of 1% cholesterol to the MUFA diet increased the LDL-cholesterol, hepatic total cholesterol, plasma triacylglycerol, and cyp7a activity regardless of the presence or absence of the CETP transgene. Dietary cholesterol decreased plasma CETP activity and liver CETP mRNA levels in CETP-TG mice fed a diet enriched in MUFA.

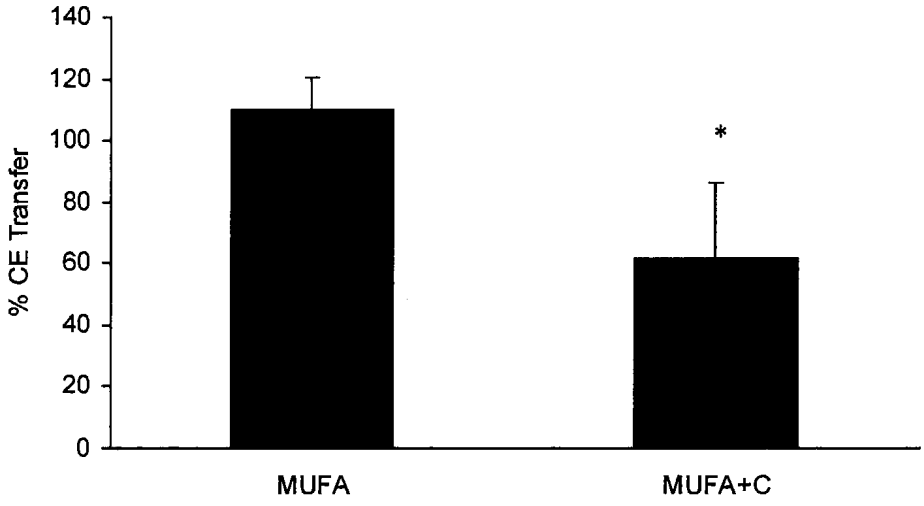


Figure 3.1: Addition of cholesterol to a diet enriched in MUFA inhibits CETP activity. The CETP transgenic mice were fed a high fat diet enriched in MUFA (20% w/w) in the presence (n=8) or absence (n=4) of 1% cholesterol. Plasma was collected and assayed for CETP activity using the radioisotope method as explained in the methods section. Values shown are mean ± standard deviation. Differences between groups were evaluated using Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

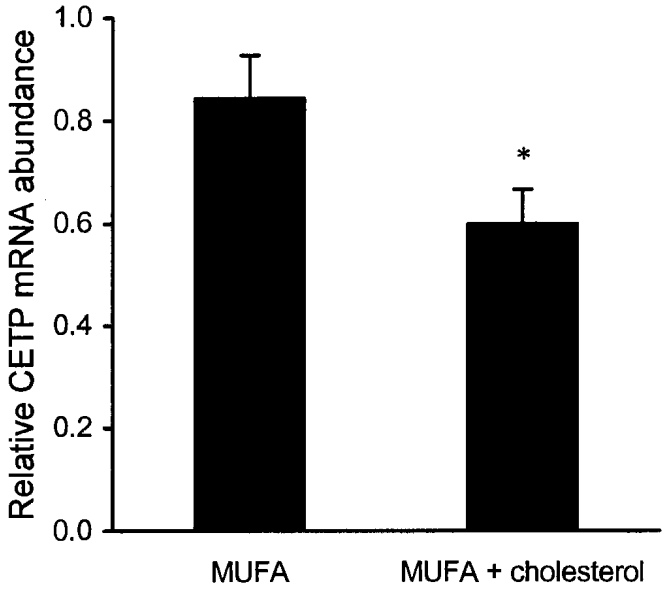


Figure 3.2: The addition of cholesterol to a diet enriched in MUFA inhibits CETP gene expression. The CETP transgenic mice were fed a high fat diet enriched in MUFA (20% w/w) in the presence (n=8) or absence (n=4) of 1% cholesterol. Total liver RNA was reverse transcribed and the cDNA template for CETP and GAPDH was amplified as described in methods. The amounts of amplified templates were quantitated and the abundance of CETP mRNA is expressed relative to GAPDH mRNA content. Values are mean ± standard deviation. Differences between groups were evaluated using Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

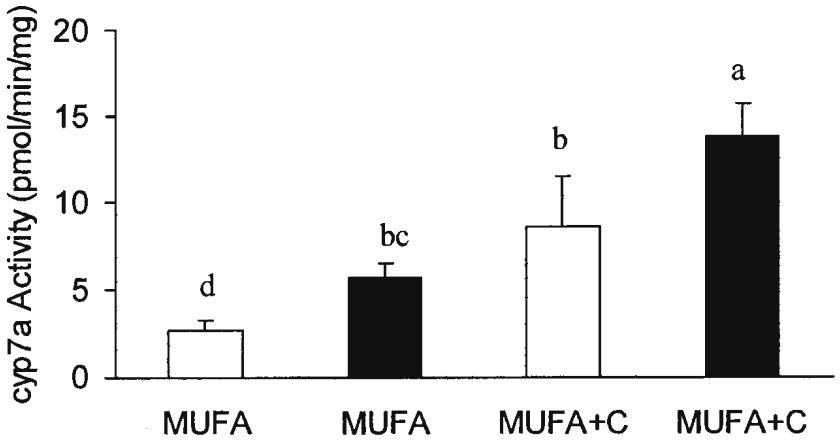


Figure 3.3: Dietary cholesterol increases cyp7a activity in CETP-TG and non-TG mice. The CETP transgenic (CETP-TG; solid bars) and non transgenic (NON-TG; open bars) mice were fed a diet enriched in MUFA (20% w/w) in the presence (MUFA + C) or absence (MUFA) of 1% cholesterol. Microsomes were prepared from liver samples and assayed for cyp7a activity as described in methods. Values shown are mean ± standard deviation (CETP-TG, MUFA n=4; CETP-TG, MUFA+C n=8; non-TG, MUFA n=8; non-TG, MUFA+C n=5). Differences between groups were tested using analysis of variance (ANOVA). Values without a common superscript are significantly different. ($p < 0.05$).

3.2 Effect of a high fat diet enriched in saturated fatty acids (SFA) on cholesterol metabolism

3.2.1 Body weight and liver weight of mice fed SFA diet

Body weights were recorded to ensure that mice were eating the experimental diets. The CETP-TG and non-TG mice fed SFA, with or without dietary cholesterol showed no significant differences in body weight over two week feeding period (Table 3.4). There were no significant differences in liver weights in CETP-TG and non-TG mice fed SFA diet with or without dietary cholesterol.

3.2.2 Plasma lipid levels

Changes in plasma lipid levels of CETP-TG and non-TG mice fed a diet enriched in SFA with or without cholesterol are shown in Table 3.5. Total plasma cholesterol concentrations were not significantly different between the CETP-TG and non-TG mice fed a diet enriched in SFA. Addition of cholesterol to the SFA diet had no significant effect on the concentrations of total plasma cholesterol concentrations in both CETP-TG and non-TG mice. The LDL-cholesterol concentrations were not significantly different between CETP-TG and non-TG mice fed a diet enriched in SFA. Addition of cholesterol to the SFA diet caused a 1.35 (p=0.01) and 1.26 fold increase (p=0.006) in LDL-cholesterol concentrations in CETP-TG and non-TG mice respectively. The HDL-cholesterol concentrations of CETP-TG mice were lower compared to non-TG mice (66% of non-TG mice fed SFA diet, p=0.04). Addition of cholesterol to the SFA diet had no significant effect on the HDL-cholesterol concentrations in both CETP-TG and non-

Table 3.4: Body weight and liver weight of CETP transgenic (CETP-TG) and non transgenic (NON-TG) mice fed SFA diet in the presence or absence of 1% cholesterol.[1]

Group	Diet	n	Original body weight (g)	Final body weight (g)	Liver weight (g)
CETP-TG	SFA	4	23.13±1.84	25.30±1.87	1.38±0.07
	SFA + cholesterol	5	23.08±3.14	25.14±3.21	1.22±0.21
NON-TG	SFA	5	23.05±2.98	24.05±2.88	1.21±0.20
	SFA + cholesterol	4	23.07±4.20	24.20±3.08	1.23±0.23

[1] The mice were weighed at the beginning and conclusion of the experiment. The livers were collected and weighed at the conclusion of the experiment. Values shown are mean ± standard deviation

Table 3.5: Plasma lipid levels of CETP transgenic (CETP-TG) and non transgenic (NON-TG) mice fed a diet enriched in SFA.[1]

Group	Diet	n	Total Cholesterol (mmol/L)	LDL-Cholesterol (mmol/L)	HDL-Cholesterol (mmol/L)	Total Triacylglycerol (mmol/L)
CETP-TG	SFA	4	1.48 ± 0.38^{a}	0.57 ± 0.03^{b}	0.59 ± 0.13^{b}	0.61 ± 0.23^{a}
	SFA + cholesterol	5	1.61 ± 0.42^{a}	0.77 ± 0.04^{a}	0.57 ± 0.12^{b}	0.72 ± 0.12^{a}
NON-TG	SFA	5	1.55 ± 0.17^{a}	0.61 ± 0.02^{b}	0.89 ± 0.12^{a}	0.64 ± 0.11^{a}
	SFA+ cholesterol	4	2.00 ± 0.42^{a}	0.77 ± 0.03^{a}	0.84 ± 0.12^{ab}	0.71 ± 0.29^{a}

[1] Values shown are mean ± standard deviation. Values without a common superscript are significantly different. ($p < 0.05$).

TG mice. Total plasma triacylglycerol concentrations were not significantly different between the CETP-TG and non-TG mice fed a diet enriched in SFA. Addition of cholesterol to the SFA diet had no significant effect on the concentrations of total plasma triacylglycerol in both CETP-TG and non-TG mice.

3.2.3 Hepatic lipid levels

Changes in hepatic lipid levels of CETP-TG and non-TG mice fed a high fat diet enriched in SFA with or without cholesterol are shown in Table 3.6. There was no significant difference in the hepatic total cholesterol concentrations of CETP-TG and non-TG mice fed a diet enriched in SFA. The addition of cholesterol to the SFA diet caused a significant increase in hepatic total cholesterol concentration in both CETP-TG and non-TG mice (2.2 fold, p=0.01 and 2 fold, p=0.001 increase respectively). There was no significant difference in hepatic total triacylglycerol concentrations of CETP-TG and non-TG mice fed a diet enriched in SFA. Addition of cholesterol to the SFA diet had no significant effect on the hepatic total triacylglycerol concentrations in both CETP-TG and non-TG mice.

3.2.4 Plasma CETP activity and hepatic CETP mRNA levels

The CETP activity and mRNA expression was undetectable in non-TG mice, thus data is only presented for CETP-TG mice. The plasma CETP activity increased with the addition of cholesterol to the SFA diet (1.7 fold, p=0.02) (Figure 3.4). However, while the hepatic CETP mRNA expression shows a trend toward an increase, there is no

Table 3.6: Hepatic lipid levels of CETP transgenic (CETP-TG) and non transgenic (NON-TG) mice fed a diet enriched in SFA.[1]

Group	Diet	n	Total Cholesterol (mmol/mg liver)	Total Triacylglycerol (mmol/mg liver)
CETP-TG	SFA	4	16.43 ± 3.37^{b}	7.19 ± 2.47^{a}
	SFA + cholesterol	5	35.27 ± 6.93^{a}	9.76 ± 2.05^{a}
NON-TG	SFA	5	16.59 ± 2.81^{b}	8.24 ± 3.00^{a}
	SFA + cholesterol	4	34.07 ± 3.76^{a}	10.14 ± 2.81^{a}

[1] Values shown are mean ± standard deviation. Values without a common superscript are significantly different. ($p < 0.05$).

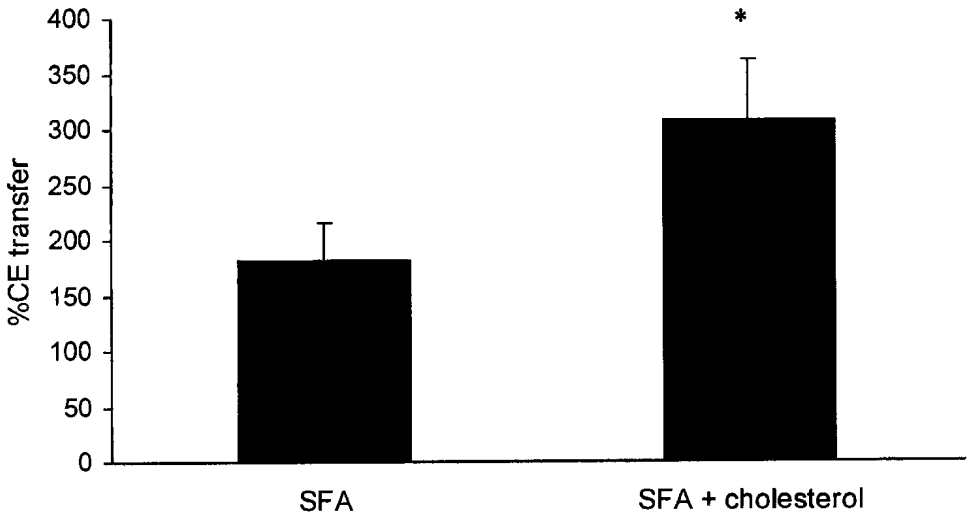


Figure 3.4: Addition of cholesterol to a diet enriched in SFA increases CETP activity. The CETP transgenic mice were fed a high fat diet enriched in SFA (20% w/w) in the presence (n=5) or absence (n=4) of 1% cholesterol. Plasma was collected and assayed for CETP activity using the radioisotope method as explained in the methods section. Values shown are the mean ± standard deviation. Differences between groups were evaluated using Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with a * if significant.

significant change with the addition of cholesterol to the SFA diet (Figure 3.5).

3.2.5 Cholesterol 7α-hydroxylase (cyp7a) activity

To investigate whether dietary cholesterol influences the breakdown of cholesterol in liver, the activity of cyp7a was measured. The cyp7a activity of CETP-TG mice fed a diet enriched in SFA was significantly lower as compared to non-TG mice fed the SFA diet (70% of non-TG mice, p=0.03) (Figure 3.6). The addition of cholesterol to the SFA diet increased cyp7a activity in both non-TG and CETP-TG mice (2.2 fold, p=0.002 and 2 fold, p=0.000008 increase respectively).

3.2.6 Summary of findings when mice were fed a diet rich in SFA

The expression of CETP gene decreased plasma HDL-cholesterol levels in mice. The addition of cholesterol to the SFA diet had no effect on plasma LDL-cholesterol concentrations, whereas the hepatic total cholesterol concentration increased. The cyp7a activity decreased in mice expressing CETP, addition of cholesterol to the SFA diet increased cyp7a activity in both CETP-TG and non-TG mice. Dietary cholesterol increased plasma CETP activity in mice fed a diet enriched in SFA but there was no corresponding increase in liver CETP mRNA.

3.3 Comparison of the effect of different dietary fats on CETP regulation

The CETP activity of CETP-TG mice fed diets enriched in SFA are significantly higher than diets enriched in MUFA (1.7 fold, p=0.03) (Figure 3.7). The CETP activity of

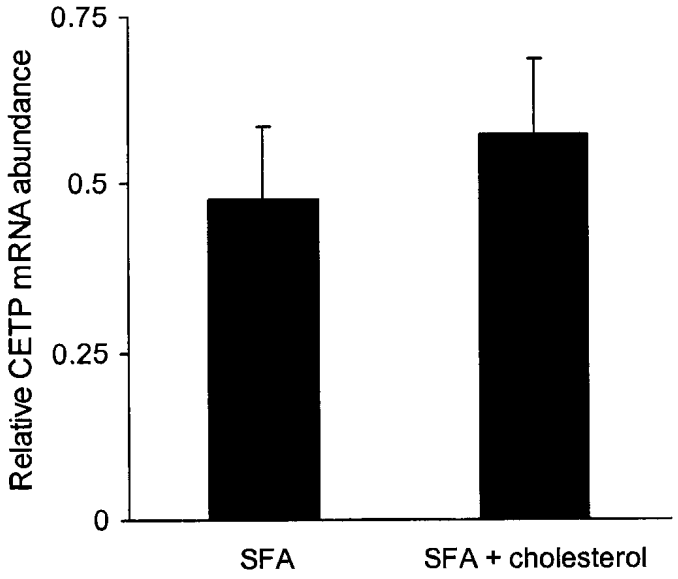


Figure 3.5: The addition of cholesterol to a diet enriched in SFA has no significant effect on CETP mRNA abundance. The CETP transgenic mice were fed a high fat diet enriched in SFA (20% w/w) in the presence (n=5) or absence (n=4) of 1% cholesterol. Total liver RNA was reverse transcribed and the cDNA template for CETP and GAPDH was amplified as described in methods. The amounts of amplified templates were quantitated and the abundance of CETP mRNA is expressed relative to GAPDH mRNA content. Values are mean ± standard deviation. Differences between groups were evaluated using Student's *t* test. Differences were considered significant if $p < 0.05$.

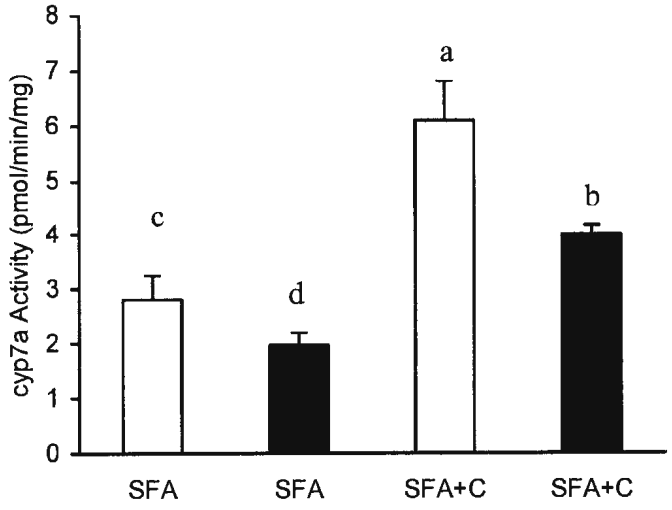


Figure 3.6: Dietary cholesterol increases cyp7a activity in CETP-TG and non-TG mice. The CETP transgenic (CETP-TG; solid bars) and non transgenic (NON-TG; open bars) mice were fed a diet enriched in SFA (20% w/w) in the presence (SFA + C) or absence (SFA) of 1% cholesterol. Microsomes were prepared from liver samples and assayed for cyp7a activity as described in methods. Values shown are mean ± standard deviation (CETP-TG, SFA n=4; CETP-TG, SFA+C n=5; non-TG, SFA n=5; non-TG, SFA+C n=4). Differences between groups were tested using analysis of variance (ANOVA). Values without a common superscript are significantly different. ($p < 0.05$).

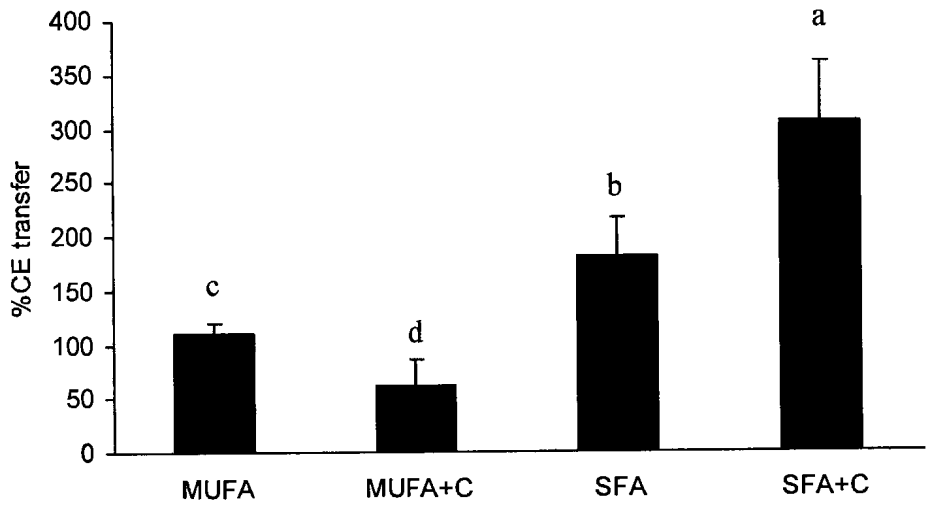


Figure 3.7: Effect of MUFA and SFA enriched diets in the presence or absence of cholesterol on CETP activity. The CETP transgenic mice were fed a high fat diet enriched in either MUFA or SFA (20% w/w) in the presence (MUFA+C, n=8; SFA+C, n=5) or absence of 1% cholesterol (MUFA, n=4; SFA, n=4). Plasma was collected and assayed for CETP activity using the radioisotope method as explained in the methods section. Values shown are mean ± standard deviation. Differences between groups were tested using analysis of variance (ANOVA). Values without a common superscript are significantly different. ($p < 0.05$).

CETP-TG mice fed a SFA diet with the addition of 1% cholesterol is significantly higher compared to mice fed a MUFA diet with the addition of 1% cholesterol (5 fold, p=0.0009). The CETP mRNA abundance of CETP-TG mice fed a MUFA enriched diet is significantly higher than that of mice fed the SFA enriched diet (1.8 fold, p=0.01)(Figure 3.8). However, there is no difference in the CETP mRNA abundance of CETP-TG mice fed MUFA and SFA diets when cholesterol is added to these diets.

In summary, comparison of the CETP-TG mice fed different fat types show that the basal CETP activity is higher when the mice are fed a diet enriched in SFA compared to mice fed a diet enriched in MUFA. There was a different response to dietary cholesterol with the basal fat type, CETP activity decreased when cholesterol was added to the MUFA diet but increased when added to the SFA diet. CETP mRNA levels were lower in animals fed the SFA diet than those fed the MUFA diet. Addition of cholesterol to the MUFA diet decreased CETP mRNA levels but there was no significant change when cholesterol was added to the SFA diet. These results suggest that the effect of dietary cholesterol on CETP depends on the composition of the dietary fat (saturated or monounsaturated). The mechanism appears to involve, at least in part, changes in the liver CETP mRNA. This leads to the study of the transcriptional control of CETP using CETP gene constructs in cell culture.

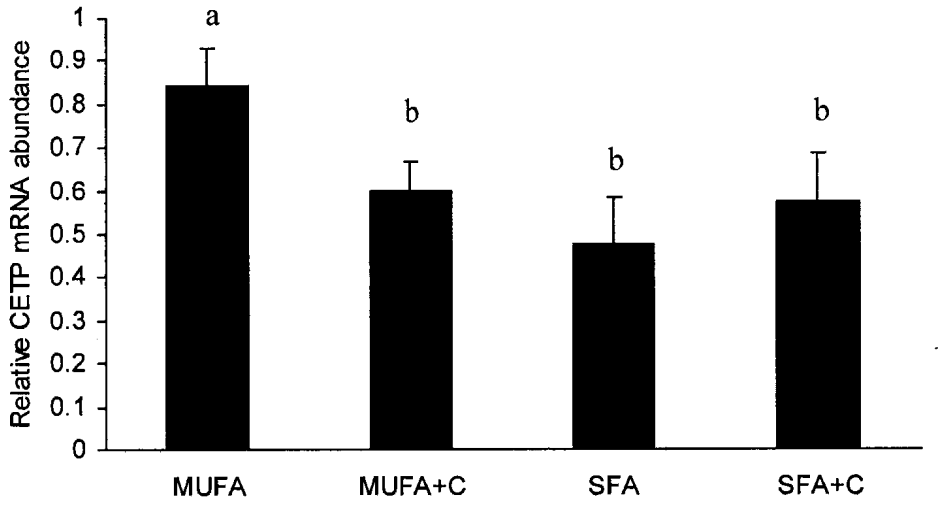


Figure 3.8: CETP mRNA abundance in CETP-TG mice fed diets enriched in various fatty acids in the presence or absence of dietary cholesterol. The CETP transgenic mice were fed a high fat diet enriched in either MUFA or SFA (20% w/w) in the presence (MUFA+C, n=8; SFA+C, n=5) or absence of 1% cholesterol (MUFA, n=4; SFA, n=4). Total liver RNA was reverse transcribed and the cDNA template for CETP and GAPDH was amplified as described in methods. The amounts of amplified templates were quantitated and the abundance of CETP mRNA is expressed relative to GAPDH mRNA content. Values are mean ± standard deviation. Differences between groups were tested using analysis of variance (ANOVA). Values without a common superscript are significantly different. ($p < 0.05$).

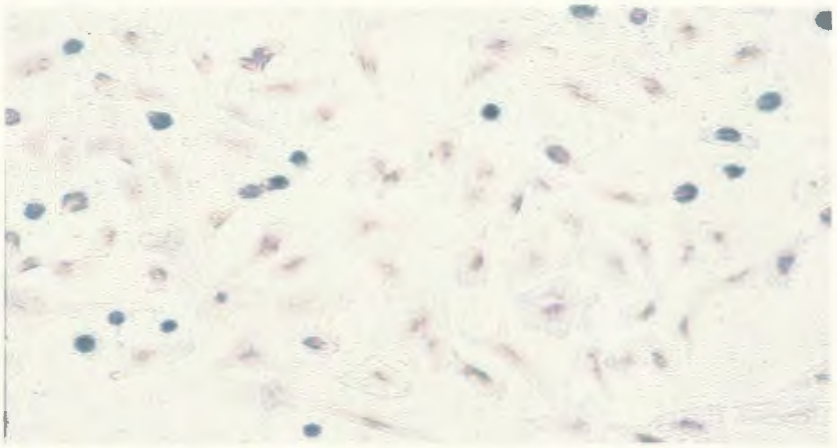
3.4 Mechanism of the human CETP gene regulation by fatty acids and cholesterol

3.4.1 The SW 872 cell line and CETP regulation

SW 872 cells, a human liposarcoma cell line, have previously been shown to synthesize and secrete CETP when grown in the presence of insulin and oleic acid (Richardson *et al.*, 1996). Addition of LDL cholesterol and 25-OH cholesterol increased the CETP activity and mRNA abundance in the SW 872 cells (Richardson *et al.*, 1996; Gauthier *et al.*, 1999). These findings suggest that SW 872 cells might be an appropriate cell line to study the regulation of CETP.

The morphology of SW 872 cells changed when grown in the presence of insulin and oleic acid for 48 hours. The cells appeared to be round as opposed to spindle shaped and fibroblast like. To investigate whether SW 872 cells accumulate lipids when grown in the presence of insulin and oleic acid, the cells were grown in differentiation medium and regular growth medium for 48 hours and stained with oil red O (Figure 3.9). The SW 872 cells grown in the presence of differentiation medium show greater lipid accumulation as judged by the red staining (Panel B) compared to the cells grown in regular growth medium (Panel A). Lipids were extracted from SW 872 cells grown in regular and differentiation medium, at different time points. Triacylglycerol and cholesterol accumulation increased when the cells are grown in the presence of differentiation medium compared to cells grown in regular growth medium (Figure 3.10 and 3.11 respectively).

A. Control



B. Differentiated

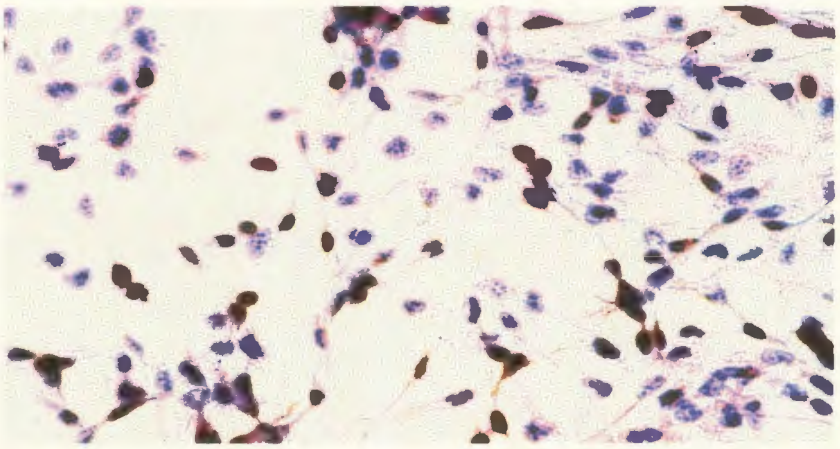


Figure 3.9: Oleic acid and insulin cause lipid accumulation in SW 872 cells. SW 872 cells were grown to 70% confluency in regular growth media. Growth media was then replaced by regular growth medium without FBS (control) or differentiation media (differentiated) for 48 h. At 48h, the cells were stained with Oil Red O as described in methods section.

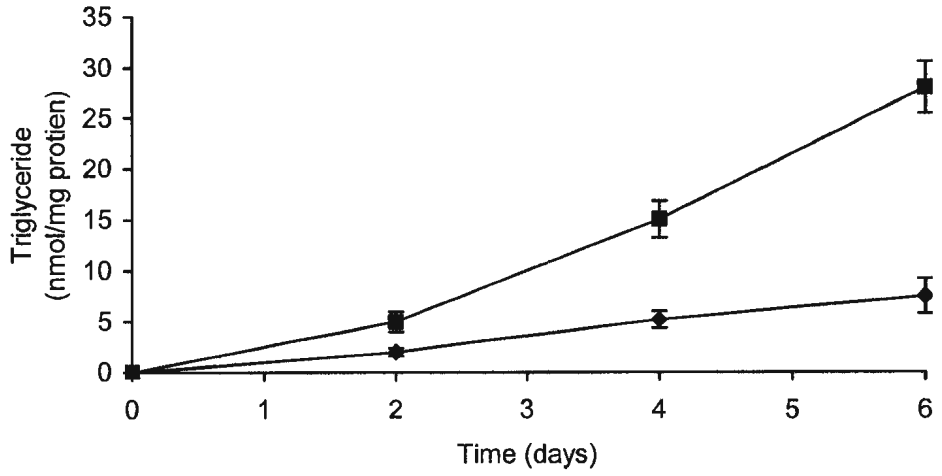


Figure 3.10: Oleic acid and insulin cause triglyceride accumulation in SW 872 cells. SW 872 cells were grown to 70% confluency in regular growth medium. Growth media was then replaced by differentiation media (differentiated, ■) or regular growth medium without FBS (control, ♦) on day 0. Lipids were extracted and assayed for triglyceride concentration at 0, 2, 4 and 6 days. Results shown represent the mean ± standard deviation.

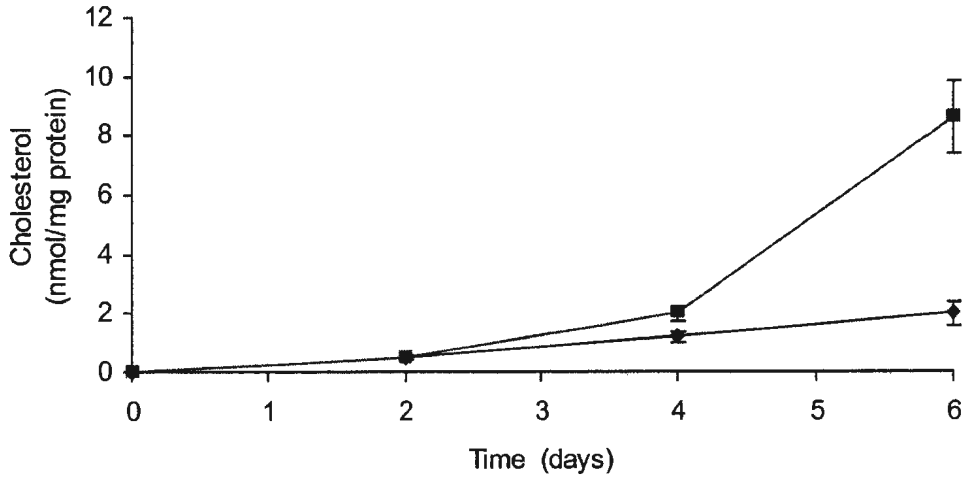


Figure 3.11: Oleic acid and insulin cause cholesterol accumulation in SW 872 cells. SW 872 cells were grown to 70% confluency in regular growth medium. Growth media was then replaced by differentiation media (differentiated, ■) or regular growth medium without FBS (control, ♦) on day 0. Lipids were extracted and assayed for cholesterol concentration at 0, 2, 4 and 6 days. Results shown represent the mean ± standard deviation.

3.4.2 Expression of CETP.CAT gene chimeras in SW 872 cells requires cotransfection of C/EBP$_\alpha$

Chimeric gene constructs harbouring sequential deletions of the human CETP gene promoter linked to CAT as a reporter (150CETP.CAT, 650CETP.CAT, 3200CETP.CAT) were transfected into SW 872 cells that had been grown in differentiation medium for 24 hours. Cells were cotransfected with β-galactosidase as a control for transfection efficiency. CETP expression was extremely low under these conditions (Figure 3.12; Panel A) as judged by the CAT activity. Since C/EBP$_\alpha$ is known to increase the expression of CETP (Agellon *et al.*, 1992), we cotransfected the C/EBP$_\alpha$ expression vector along with the CETP chimeric gene constructs (Figure 3.12, Panel A). Cotransfection of the C/EBP$_\alpha$ expression vector significantly increased the expression of 150CETP.CAT and 650CETP.CAT (4.5 fold, p=0.04 and 3.3 fold p=0.007 increase respectively) but had little effect on the expression of 3200CETP.CAT (Figure 3.12, Panel B). As C/EBP$_\alpha$ was found to be required for CETP expression in SW 872 cells, C/EBP$_\alpha$ was cotransfected with the CETP.CAT gene constructs in all further experiments

3.4.3 Regulation of the human CETP gene by 25-OH cholesterol is via LXR$_\alpha$/RXR$_\alpha$

Cholesterol mediated stimulation of the CETP gene is via LXR$_\alpha$/RXR$_\alpha$ and is contained within 400 bp of the promoter region (Luo *et al.*, 2000). To confirm these previous findings in SW 872 cells, differentiated SW 872 cells were transfected with

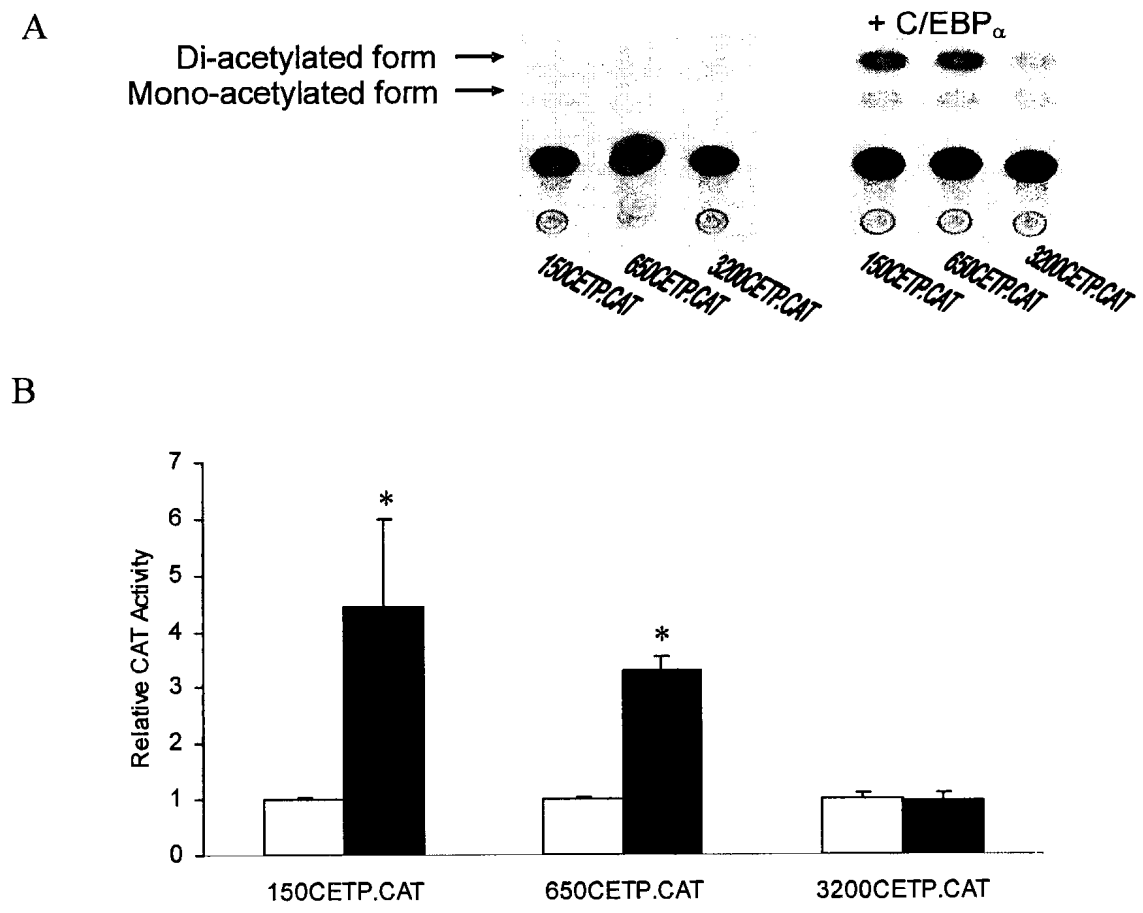


Figure 3.12: The expression of CETP is enhanced by coexpressing C/EBP$_\alpha$. The CETP.CAT gene chimeras (panel A; panel B, open bars) or CETP.CAT gene chimeras and a plasmid encoding C/EBP$_\alpha$ (panel A; panel B, solid bars) were cotransfected into differentiated SW 872 cells. The CAT activity was normalized to the β-galactosidase activity encoded by the β-galactosidase expression vector. The normalized CAT activity in the control cells was assigned a value of one. Results shown represent the mean ± standard deviation of three independent experiments. Differences were evaluated using the Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

CETP chimeric gene constructs along with LXR_α and RXR_α expression vectors and β-galactosidase (Figure 3.13). The transfected cells were treated with 4 μg/ml of 25-OH cholesterol. The expression of 650CETP.CAT was increased 3.3 fold ($p \leq 0.002$) when 25-OH cholesterol was added to the growth media. There was no significant effect on the expression of 150CETP.CAT or 3200CETP.CAT by the addition of 25-OH cholesterol. This cholesterol mediated induction was not observed in cells when LXR_α and RXR_α is not cotransfected along with the CETP chimeric gene constructs (data not shown). These findings are similar to the previous observations by Luo *et al.* (2000) suggesting that the cholesterol mediated induction of CETP by LXR_α/RXR_α is contained within 650 bp of the promoter.

3.4.4 Effect of oleic acid on the regulation of human CETP gene expression

Oleic acid at a concentration of 50 μM had no effect on the expression of 150CETP.CAT, 650CETP.CAT, and 3200CETP.CAT when the differentiated SW 872 cells are transfected with CETP chimeric gene constructs and β-galactosidase only (Figure 3.14). Fatty acids regulate gene expression of various enzymes via $PPAR_\alpha/RXR_\alpha$. Thus, we co-expressed $PPAR_\alpha$ and RXR_α along with the CETP chimeric gene constructs in differentiated SW 872 cells to investigate whether expression of $PPAR_\alpha$ and RXR_α mediates the regulation of CETP by fatty acids. Treatment of differentiated SW 872 cells with 50μM oleic acid resulted in a 1.4 fold increase (p=0.006) in the expression of 650CETP.CAT (Figure 3.15). However, there was no significant change in the expression of 150CETP.CAT and 3200CETP.CAT. This suggests that

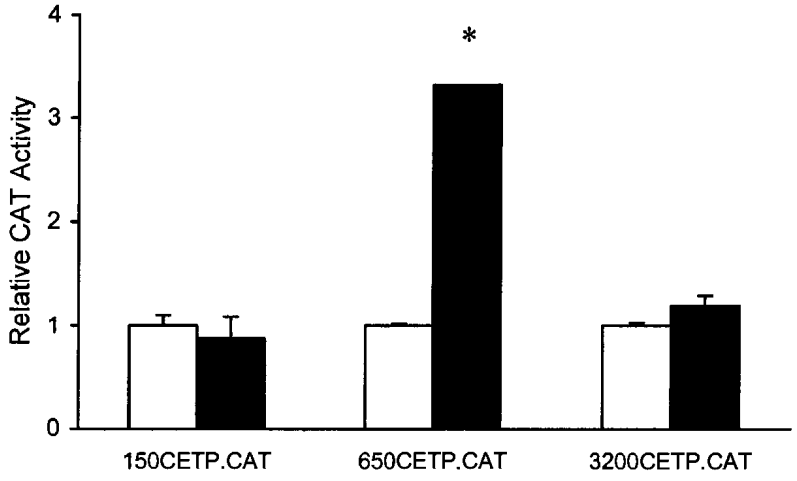


Figure 3.13: Regulation of CETP gene by 25-OH cholesterol via LXR_α/RXR_α. The CETP.CAT gene chimeras and plasmids encoding C/EBP_α LXR_α and RXR_α were cotransfected into differentiated SW 872 cells. 25-OH cholesterol was added to the culture medium to a final concentration of 4 μg/ml (solid bars). Control cells received DMSO alone (open bars). The CAT activity was normalized to the β-galactosidase activity encoded by the β-galactosidase expression vector. The normalized CAT activity in the control cells was assigned a value of one. Results shown represent the mean ± standard deviation of three independent experiments. Differences were evaluated using the Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

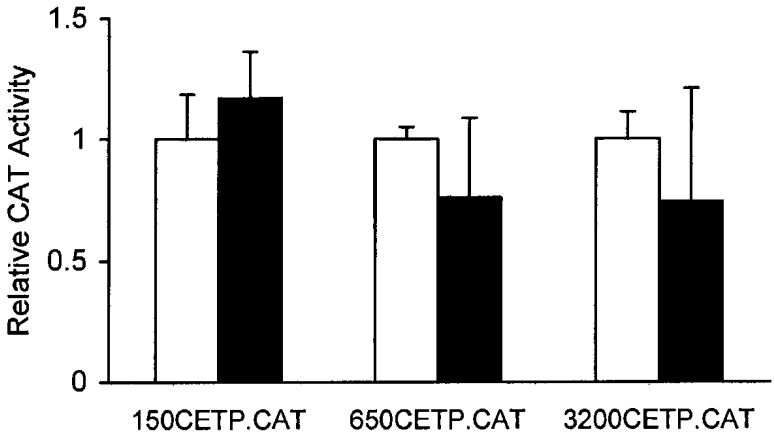


Figure 3.14: Regulation of human CETP gene by oleic acid. The CETP.CAT gene chimeras and a plasmid encoding C/EBP$_\alpha$ were cotransfected into differentiated SW 872 cells. Oleic acid (18:1) complexed to fatty acid free BSA was added to the culture medium to a final concentration of 50 μM (solid bars). Control cells received BSA alone (open bars). The CAT activity was normalized to the β-galactosidase activity encoded by the β-galactosidase expression vector. The normalized CAT activity in the control cells was assigned a value of one. Results shown represent the mean ± standard deviation of three independent experiments. Differences were evaluated using the Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

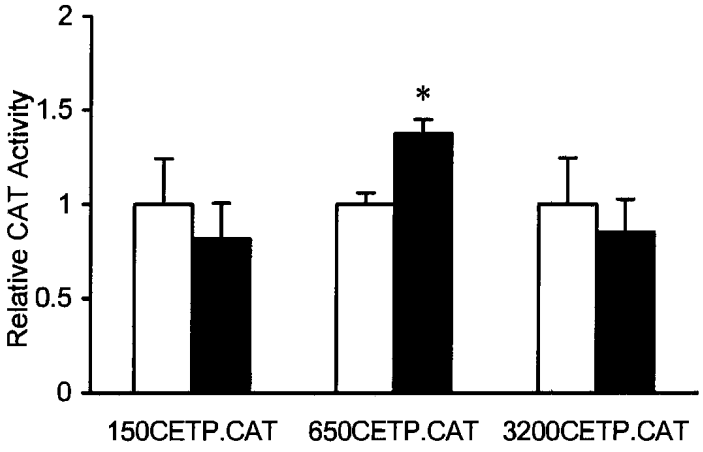


Figure 3.15: The regulation of CETP gene by oleic acid is mediated via PPAR$_\alpha$/RXR$_\alpha$. The CETP.CAT gene chimeras and plasmids encoding C/EBP$_\alpha$, PPAR$_\alpha$ and RXR$_\alpha$ were cotransfected into differentiated SW 872 cells. Oleic acid (18:1) complexed to fatty acid free BSA was added to the culture medium to a final concentration of 50 μM (solid bars). Control cells received BSA alone (open bars). The CAT activity was normalized to the β-galactosidase activity encoded by the β-galactosidase expression vector. The normalized CAT activity in the control cells was assigned a value of one. Results shown represent the mean ± standard deviation of three independent experiments. Differences were evaluated using the Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

$PPAR_\alpha/RXR_\alpha$ mediates the effect of oleic acid on the CETP gene expression and this interaction occurs within the -650 bp region of the 5' regulatory region.

As oleic acid and cholesterol regulate the human CETP gene independently when cotransfected with $PPAR_\alpha/RXR_\alpha$ or LXR_α/RXR_α, we further investigated whether cholesterol and fatty acids given in combination have a similar effect on CETP gene expression. When SW 872 cells are cotransfected with $PPAR_\alpha$ and RXR_α and treated with 4 μg/ml of 25-OH cholesterol and 50μM oleic acid together, there was a significant increase in the expression of 3200CETP.CAT and 650CETP.CAT (Figure 3.16). However, there was no significant effect on the expression of 150CETP.CAT. On the other hand, when SW 872 cells were cotransfected with LXR_α and RXR_α and treated with 4 μg/ml of 25-OH cholesterol and 50 μM oleic acid, there was a significant decrease in the expression of 150CETP.CAT (54% of the control, p=0.006), and a significant increase in the expression of 650CETP.CAT (3.2 fold, p=0.02) and 3200CETP.CAT (2.5 fold, p=0.04) (Figure 3.17).

Next, we co-transfected the CETP.CAT gene chimeras with $PPAR_\alpha$, RXR_α and LXR_α and treated the cells with oleic acid and cholesterol to investigated whether these transcription factors interact to mediate the regulation of CETP. The addition of 50 μM oleic acid and 4 μg/mL 25-OH cholesterol to differentiated SW 872 cells coexpressing LXR_α, $PPAR_\alpha$, and RXR_α decreased the expression of 150CETP.CAT (83% of control, p=0.02) but increased the expression of 3200CETP.CAT (1.7 fold, p=0.03) (Figure 3.18) with no significant effect on 650CETP.CAT expression.

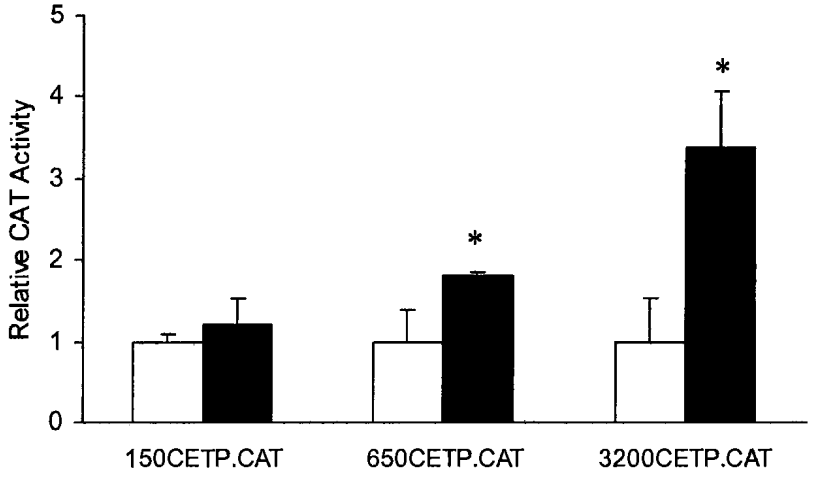


Figure 3.16: Regulation of CETP gene by oleic acid and 25-OH cholesterol via PPAR$_\alpha$/RXR$_\alpha$. The CETP.CAT gene chimeras and plasmids encoding C/EBP$_\alpha$, PPAR$_\alpha$ and RXR$_\alpha$ were cotransfected into differentiated SW 872 cells. Oleic acid (18:1) complexed to fatty acid free BSA was added to the culture medium to a final concentration of 50 μM and 25-OH cholesterol was added to the culture medium to a final concentration of 4 μg/ml (solid bars). Control cells received vehicle alone (open bars). The CAT activity was normalized to the β-galactosidase activity encoded by the β-galactosidase expression vector. The normalized CAT activity in the control cells was assigned a value of one. Results shown represent the mean ± standard deviation of three independent experiments. Differences were evaluated using the Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

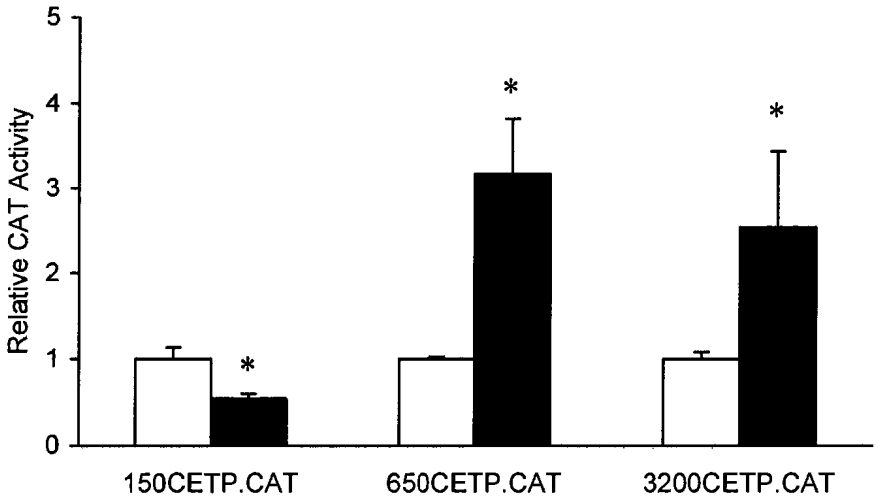


Figure 3.17: Regulation of CETP gene by oleic acid and 25-OH cholesterol via LXR_α/RXR_α. The CETP.CAT gene chimeras and plasmids encoding C/EBP_α, LXR_α and RXR_α were cotransfected into differentiated SW 872 cells. Oleic acid (18:1) complexed to fatty acid free BSA was added to the culture medium to a final concentration of 50 μM and 25-OH cholesterol was added to the culture medium to a final concentration of 4 μg/ml (solid bars). Control cells received vehicle alone (open bars). The CAT activity was normalized to the β-galactosidase activity encoded by the β-galactosidase expression vector. The normalized CAT activity in the control cells was assigned a value of one. Results shown represent the mean ± standard deviation of three independent experiments. Differences were evaluated using the Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

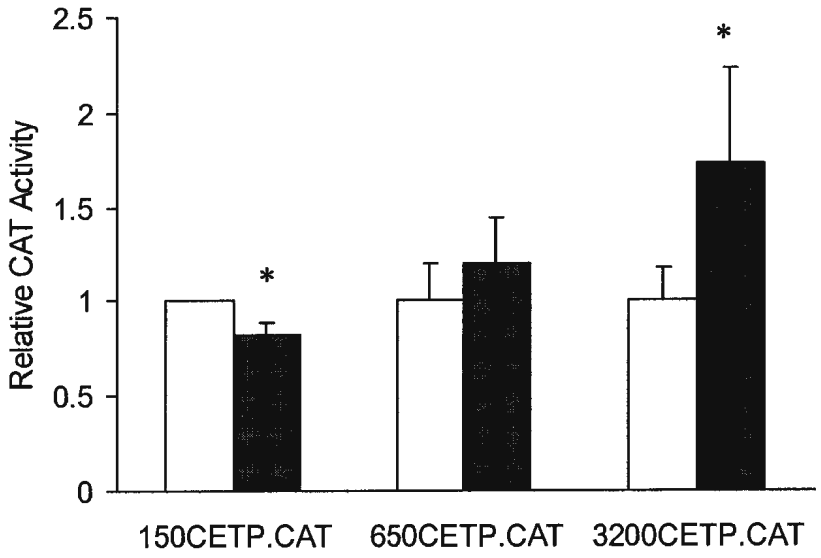


Figure 3.18: Regulation of CETP gene by oleic acid and 25-OH cholesterol via PPAR$_\alpha$/RXR$_\alpha$/LXR$_\alpha$. The CETP.CAT gene chimeras and plasmids encoding C/EBP$_\alpha$, PPAR$_\alpha$, LXR$_\alpha$ and RXR$_\alpha$ were cotransfected into differentiated SW 872 cells. Oleic acid (18:1) complexed to fatty acid free BSA was added to the culture medium to a final concentration of 50 μM and 25-OH cholesterol was added to the culture medium to a final concentration of 4 μg/mL (solid bars). Control cells received vehicle alone (open bars). The CAT activity was normalized to the β-galactosidase activity encoded by the β-galactosidase expression vector. The normalized CAT activity in the control cells was assigned a value of one. Results shown represent the mean ± standard deviation of three independent experiments. Differences were evaluated using the Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

3.4.5 Effect of stearic acid on the regulation of human CETP gene expression

Treatment of differentiated SW 872 cells with 50 μM stearic acid (18:0) inhibited the expression of 650CETP.CAT when $PPAR_\alpha$ and RXR_α are coexpressed along with CETP.CAT gene chimeras (Figure 3.19). However, there was no significant change in the expression of 150CETP.CAT and 3200CETP.CAT.

Next, stearic acid and cholesterol were added in combination to differentiated SW 872 cells to study their combined effect. The addition of 4 μg/ml 25-OH cholesterol and 50 μM stearic acid to differentiated SW 872 cells coexpressing $PPAR_\alpha$ and RXR_α caused a decrease in the expression of 150CETP.CAT and 650CETP CAT (Figure 3.20). However, there was no significant change in 3200CETP.CAT expression.

The addition of 4 μg/ml of 25-OH cholesterol and 50 μM stearic acid to growth media of differentiated SW 872 cells co-expressing LXR_α and RXR_α caused an ~50% (p=0.03) decrease in the expression of 3200CETP.CAT (Figure 3.21). However, it had no effect on the expression of 150CETP.CAT and 650CETP.CAT.

Next, we cotransfected the SW 872 cells with $PPAR_\alpha$, RXR_α and LXR_α to investigate whether these transcription factors interact to mediate the effects of stearic acid and cholesterol on CETP gene regulation. The addition of 50 μM stearic acid and 4 μg/mL 25-OH cholesterol to differentiated SW 872 cells decrease the expression of 150CETP.CAT and 650CETP.CAT (20% of control, p=0.003; 51% of control, p=0.01 respectively) but had no effect on 3200CETP.CAT expression (Figure 3.22).

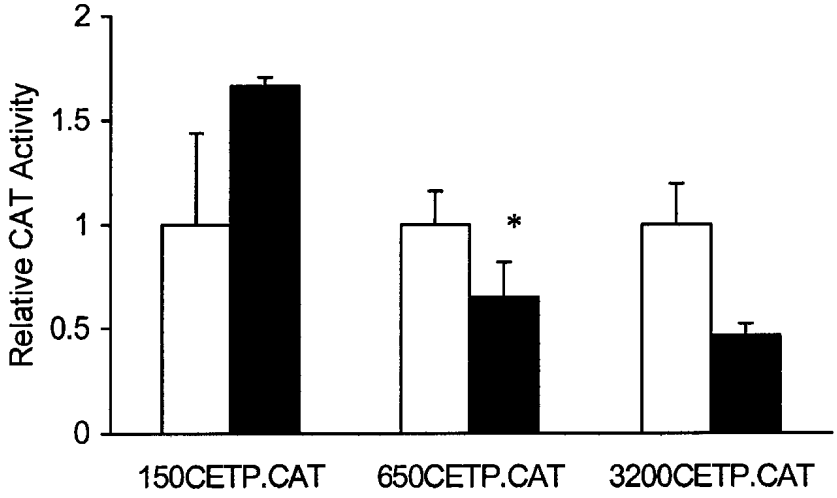


Figure 3.19: Regulation of CETP gene by stearic acid via PPAR$_\alpha$/RXR$_\alpha$. The CETP.CAT gene chimeras and plasmids encoding C/EBP$_\alpha$, PPAR$_\alpha$, and RXR$_\alpha$ were cotransfected into differentiated SW 872 cells. Stearic acid was added to the culture medium to a final concentration of 50 μM (solid bars). Control cells received BSA alone (open bars). The CAT activity was normalized to the β-galactosidase activity encoded by the β-galactosidase expression vector. The normalized CAT activity in the control cells was assigned a value of one. Results shown represent the mean ± standard deviation of three independent experiments. Differences were evaluated using the Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

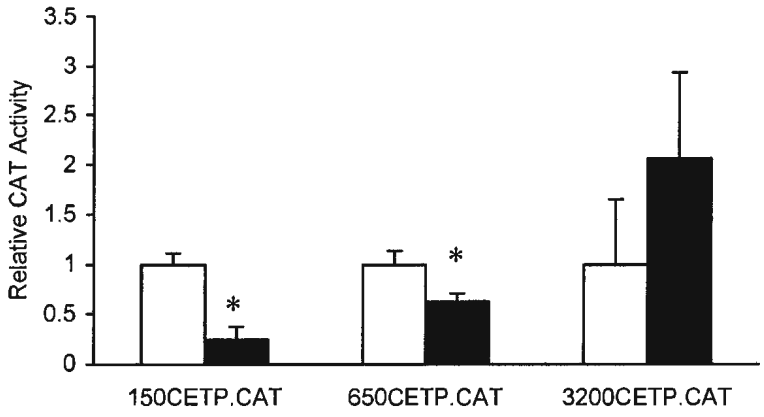


Figure 3.20: Regulation of the CETP gene by stearic acid and 25-OH cholesterol mediated via $PPAR_\alpha/RXR_\alpha$. The CETP.CAT gene chimeras and plasmids encoding C/EBP_α, $PPAR_\alpha$, and RXR_α were cotransfected into differentiated SW 872 cells. Stearic acid (18:0) complexed to fatty acid free BSA was added to the culture medium to a final concentration of 50 μM, and 25-OH cholesterol was added to the culture medium to a final concentration of 4 μg/ml (solid bars). Control cells received the vehicle alone (open bars). The CAT activity was normalized to the β-galactosidase activity encoded by the β-galactosidase expression vector. The normalized CAT activity in the control cells was assigned a value of one. Results shown represent the mean ± standard deviation of three independent experiments. Differences were evaluated using the Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

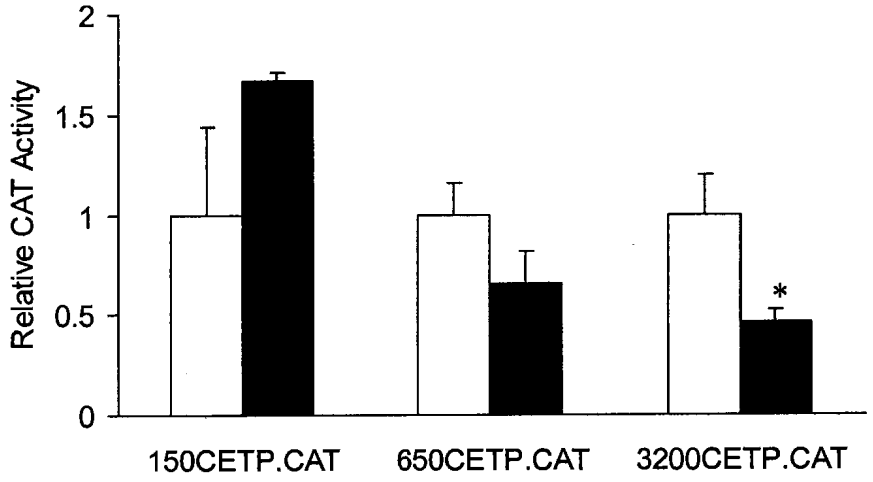


Figure 3.21: Regulation of CETP gene by stearic acid and 25-OH cholesterol via LXR_α/RXR_α. The CETP.CAT gene chimeras and plasmids encoding C/EBP_α, LXR_α, and RXR_α were cotransfected into differentiated SW 872 cells. Stearic acid (18:0) complexed to fatty acid free BSA was added to the culture medium to a final concentration of 50 μM and 25-OH cholesterol was added to the culture medium to a final concentration of 4 μg/ml (solid bars). Control cells received the vehicle alone (open bars). The CAT activity was normalized to the β-galactosidase activity encoded by the β-galactosidase expression vector. The normalized CAT activity in the control cells was assigned a value of one. Results shown represent the mean ± standard deviation of three independent experiments. Differences were evaluated using the Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

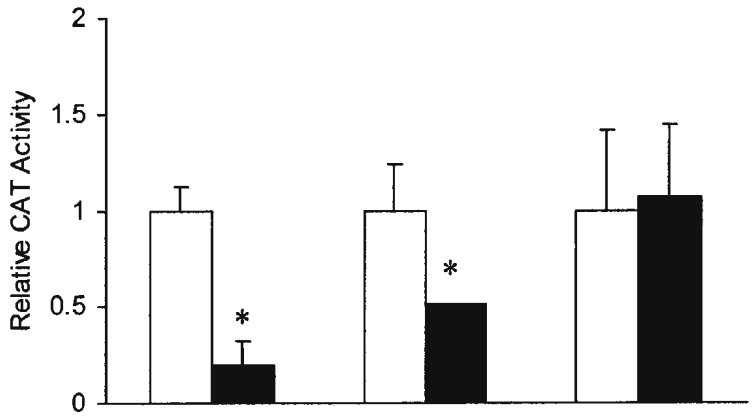


Figure 3.22: Regulation of the CETP gene by stearic acid and 25-OH cholesterol mediated via PPAR$_\alpha$/RXR$_\alpha$/LXR$_\alpha$. The CETP.CAT gene chimeras and plasmids encoding C/EBP$_\alpha$, LXR$_\alpha$, PPAR$_\alpha$ and RXR$_\alpha$ were cotransfected into differentiated SW 872 cells. Stearic acid (18:0) complexed to fatty acid free BSA was added to the culture medium to a final concentration of 50 μM and 25-OH cholesterol was added to the culture medium to a final concentration of 4 μg/mL (solid bars). Control cells received vehicle alone (open bars). The CAT activity was normalized to the β-galactosidase activity encoded by the β-galactosidase expression vector. The normalized CAT activity in the control cells was assigned a value of one. Results shown represent the mean ± standard deviation of three independent experiments. Differences were evaluated using the Student's *t* test. Differences were considered significant if $p < 0.05$ and marked with * if significant.

Chapter 4: Discussion

4.1 Effect of a high fat diet enriched in MUFA on cholesterol metabolism

4.1.1 Plasma lipid concentrations

The plasma total cholesterol concentrations of CETP-TG mice were significantly lower than those of the non-TG mice fed a diet enriched in MUFA (Table 3.2). A similar decrease in total plasma cholesterol was seen in CETP-TG mice with the CETP gene under control of the mouse metallothionein-I promoter, compared to non-TG mice fed a chow diet and a high fat diet (Agellon *et al.*, 1991). This decrease was also seen in other studies where the CETP gene was under the control of its NFR in CETP-TG mice fed a chow diet (Masucci-Magoulas *et al.*, 1996; Foger *et al.*, 1999). The decrease in plasma total cholesterol is due to a decrease in HDL-cholesterol caused by CETP transferring cholesterol esters from HDL-cholesterol to LDL- and VLDL-cholesterol. The addition of cholesterol to the MUFA enriched diet had no significant effect on total plasma cholesterol concentrations of CETP-TG and non-TG mice (Table 3.2). Feeding a cholesterol enriched diet to C57BL/6 mice has previously been shown not to have an effect on total plasma cholesterol concentrations (Schwartz *et al.* 2001). The response of different individuals and different species to dietary cholesterol varies widely due to several reasons (Beynen *et al.*, 1987). One possibility is increased breakdown of cholesterol to bile acids via cyp7a, which is induced by dietary cholesterol (Russell *et al.*, 1992). The addition of cholesterol to MUFA diet was found to induce cyp7a activity in CETP-TG and non-TG mice. Thus it is likely that increased breakdown of cholesterol to bile acids in mice fed dietary cholesterol prevents an increase in plasma total cholesterol concentrations.

There was no difference in LDL-cholesterol concentrations between CETP-TG and non-TG mice fed a MUFA enriched diet (Table 3.2). The VLDL+LDL-cholesterol in CETP-TG and non-TG mice were also not found to be significantly different when fed a chow diet or a high fat diet (Agellon *et al.*, 1991). However, they found a decrease in the ratio of free cholesterol to cholesterol ester in all lipoprotein fractions of CETP-TG mice suggesting an increase in LCAT activity to replace cholesterol ester transferred out of HDL into the apo-B lipoproteins. The addition of cholesterol to the MUFA enriched diet caused an increase in LDL-cholesterol concentrations in CETP-TG and non-TG mice (Table 3.2). The increase in LDL-cholesterol with the addition of cholesterol to the diet is expected as a high fat, high cholesterol diet is known to increase LDL+VLDL-cholesterol in C57/BL6 mice (LeBoeuf *et al.*, 1990).

HDL-cholesterol was lower in CETP-TG mice compared to the non-TG mice when fed a MUFA-enriched diet (Table 3.2). The decrease in HDL-cholesterol is the result of CETP transferring cholesterol esters from HDL-cholesterol to LDL- and VLDL-cholesterol (Agellon *et al.* 1991; Foger *et al.*, 1999; Kawano *et al.*, 2000). The addition of cholesterol to the MUFA diet had no effect on HDL-cholesterol concentrations in CETP-TG and non-TG mice (Table 3.2). This is likely due to short term feeding of cholesterol (two weeks) since most studies observed changes in HDL-cholesterol by dietary cholesterol after four or more weeks (Kurushima *et al.*, 1995a; Foger *et al.*, 1999). In fact CETP-TG and C57BL/6 mice fed a high fat, high cholesterol (1%) diet for 28 weeks showed an increased in HDL-cholesterol that gradually increased during the course of the feeding study (Marotti *et al.*, 1993).

Total plasma triacylglycerol concentrations were not significantly different between the CETP-TG and non-TG mice (Table 3.2). CETP transfers triacylglycerol from LDL and VLDL to HDL, but does not affect the total concentration of triacylglycerol in plasma (Chang *et al.*, 2001; Foger *et al.*, 1999).

4.1.2 Hepatic lipid concentrations

Hepatic total cholesterol concentrations between CETP-TG and non-TG mice when fed a MUFA enriched diet were not significantly different (Table 3.3). However, Chang *et al.* (2001) found an increase in hepatic total cholesterol in CETP-TG mice with the human CETP gene under the control of the apolipoprotein AI promoter compared to non-TG mice when fed a MUFA enriched diet for five weeks. Our results show a trend towards an increase but the increase was not significant. The addition of cholesterol to the MUFA diet caused a significant increase in hepatic total cholesterol concentrations for both CETP-TG and non-TG mice (Table 3.3). These results indicate that dietary cholesterol is absorbed and delivered to the liver.

Hepatic total triacylglycerol concentrations of CETP-TG mice were higher than non-TG mice when fed a diet enriched in MUFA (Table 3.3). The addition of cholesterol to the MUFA diet caused an increase in hepatic total triacylglycerol concentrations for both CETP-TG and non-TG mice (Table 3.3). This increase might be due to increased hepatic biosynthesis of triacylglycerol or decreased fatty acid oxidation (Fungwe *et al.*, 1993) and needs to be explored in future studies.

4.1.3 Plasma CETP activity and hepatic mRNA concentration

Plasma CETP activity (Figure 3.1) and hepatic CETP mRNA expression (Figure 3.2) decreased in CETP-TG mice when cholesterol was added to a diet enriched in MUFA. This differs from previous observations where addition of cholesterol to a high fat diet enriched with coconut oil, rich in 16:0 fatty acids, increased CETP mRNA and CETP activity in CETP-TG mice (Jiang *et al.*, 1992). However, Kurushima *et al* (1995a) found that addition of 5% oleic acid to a cholesterol supplemented diet in hamsters prevents cholesterol induced increase in CETP activity, whereas palmitic acid increased CETP activity (Kurushima *et al.*, 1995b). These findings support our results suggesting that oleic acid interferes with the cholesterol mediated induction of CETP activity and gene expression.

4.1.4 Cholesterol 7α–hydroxylase (cyp7a) activity

As addition of cholesterol to the MUFA diet was found to inhibit CETP activity, we used cyp7a activity that is known to be induced by dietary cholesterol as a positive control to ensure the mice were responding to dietary cholesterol. Moreover, the CETP and cyp7a promoters have similar sterol dependent induction by LXR and LRH, thus the expression of CETP and cyp7a may be coordinately regulated in the liver (Luo *et al.* 2001).

The cyp7a activity was higher in CETP-TG mice compared to non-TG mice when both species were fed a diet enriched in MUFA (Figure 3.3). CETP expression may increase reverse cholesterol transport delivering more cholesterol to the liver, which

increases cyp7a activity. The addition of cholesterol to the MUFA diet increased cyp7a activity in both non-TG and CETP-TG mice (Figure 3.3) suggesting that the mice are responding to dietary cholesterol as expected.

4.2 Effect of a high fat diet enriched in SFA on cholesterol metabolism

4.2.1 Plasma lipid concentrations

There was no difference between total plasma cholesterol concentrations in the CETP-TG and non-TG mice fed a diet enriched in SFA (Table 3.5). Previous studies by Agellon *et al.* (1991) and our data on CETP-TG mice fed the MUFA diet showed a decrease in plasma total cholesterol concentrations when compared to non-TG mice, and this decrease was mainly due to a decrease in HDL-cholesterol concentrations. Chang *et al.* (2001) found no difference in the total plasma cholesterol concentrations of transgenic mice expressing human CETP, where the CETP gene is linked to the apolipoprotein A-I promoter and non-TG mice fed diets enriched in SFA. The absence of the total plasma cholesterol lowering effect in CETP-TG mice fed a SFA diet compared to those fed a MUFA and PUFA diet could be the result of the SFA's inability to maintain hepatic LDL receptor activity (Chang *et al.*, 2001; Bucci *et al.*, 1998).

The addition of cholesterol to the SFA diet had no significant effect on the total plasma cholesterol concentrations in both CETP-TG and non-TG mice (Table 3.5). On the other hand, the LDL-cholesterol concentrations were significantly increased when cholesterol was added to the SFA diet for both CETP-TG and non-TG mice. The increase in LDL-cholesterol with the addition of cholesterol to the diet is expected as dietary

cholesterol is known to increase LDL+VLDL-cholesterol (Grundy *et al.*, 1990; LeBoeuf *et al.*, 1990).

Total plasma triacylglycerol concentrations were not significantly different between the CETP-TG and non-TG mice fed a diet enriched in SFA (Table 3.5). We had similar observations when CETP-TG and non-TG mice were fed a diet enriched in MUFA (Table 3.2). The addition of cholesterol to the SFA diet had no significant effect on the concentrations of total plasma triacylglycerol in CETP-TG and non-TG mice (Table 3.5), similar to the observations made for the CETP-TG and non-TG mice fed the MUFA diet (Table 3.2).

4.2.2 Hepatic lipid concentrations

Hepatic total cholesterol concentrations of CETP-TG and non-TG mice when fed a diet enriched in SFA were not significantly different (Table 3.6). No difference was observed in hepatic total cholesterol between CETP-TG and non-TG mice when fed a diet enriched in MUFA (Table 3.3). The addition of cholesterol to the SFA diet caused a significant increase in hepatic total cholesterol concentration for both CETP-TG and non-TG mice (Table 3.6), similar to the findings when these animals were fed a diet enriched in MUFA, indicating that dietary cholesterol is absorbed and delivered to the liver.

Hepatic total triacylglycerol concentrations of CETP-TG and non-TG mice were not different when fed a SFA enriched diet (Table 3.6). The addition of cholesterol to the SFA diet had no significant effect on the concentrations of hepatic total triacylglycerol in both CETP-TG and non-TG mice. These results differ from the MUFA study where

CETP-TG mice had higher hepatic total triacylglycerol concentrations than non-TG mice, and the addition of cholesterol caused an increase in hepatic total triacylglycerol concentrations for both CETP-TG and non-TG mice (Table 3.3). It is possible that mice like hamsters fed a SFA diet with supplemented cholesterol package more triacylglycerols into VLDL that is secreted from the liver (Fungwe *et al.*, 1994). We did not measure VLDL concentrations in these mice; however, there was a significant increase in LDL-cholesterol concentrations which might be as a result of an increase in VLDL.

4.2.3 Plasma CETP activity and hepatic CETP mRNA levels

Plasma CETP activity increased when cholesterol was added to the SFA diet (Figure 3.4). The hepatic CETP mRNA levels showed a trend towards an increase but the effect was not significant (Figure 3.5). This differs from the MUFA diet study where a significant decrease in CETP activity and hepatic CETP mRNA expression was seen with the addition of dietary cholesterol. Previous reports indicate an increase in CETP activity and mRNA abundance with the addition of dietary cholesterol. The effect is further enhanced when cholesterol is added to a diet rich in saturated fatty acids (Jiang *et al.* 1992). We found no significant increase in hepatic mRNA levels with the addition of cholesterol to the SFA diet, thus the increase in plasma CETP activity might be due to an increase in CETP secretion.

4.2.4 Cholesterol 7α hydroxylase (cyp7a) activity

Dietary cholesterol is known to raise the cyp7a activity (Russell *et al.*, 1992). We measured the cyp7a activity in CETP-TG and non-TG mice to ensure that the mice are responding to dietary cholesterol and to further investigate whether there is coordinated regulation of cyp7a and CETP. The addition of cholesterol to the SFA diet increased cyp7a activity in both non-TG and CETP-TG mice (Figure 3.6) confirming the response of cyp7a to dietary cholesterol.

The cyp7a activity is lower in CETP-TG mice compared to the non-TG mice fed SFA alone or with added cholesterol. These findings are different from mice fed the MUFA diet where CETP-TG mice showed higher cyp7a activity suggesting that more cholesterol is returned to the liver via reverse cholesterol transport. Feeding diets enriched in saturated fatty acids are known to inhibit LDL-receptors (Bucci *et al.*, 1992), thus under these conditions cholesterol might not be returned to the liver as efficiently. Kurushima *et al.*, (1995b) found higher CETP activity in hamsters fed a diet enriched in SFA compared to a diet enriched in MUFA. However, the cyp7a activity was lower in SFA fed animals similar to our observations. Thus, CETP and cyp7a might not be coordinately regulated under certain dietary conditions.

4.3 Mechanisms of the regulation of the human CETP gene by fatty acids and cholesterol

The hepatic CETP mRNA expression of CETP-TG mice fed the MUFA enriched diet was significantly higher than mice fed the SFA enriched diet, indicating that the type

of fat itself has significant effect on the regulation of CETP gene expression (Figure 3.8). The following sections deal with the molecular mechanisms involved in fatty acid and cholesterol mediated regulation of CETP.

4.3.1 Lipid accumulation in SW 872 cells

The SW 872 cells were chosen to study CETP gene regulation as this cell line synthesizes and secretes CETP (Richardson *et al.*, 1996), and expresses the necessary factors for CETP regulation by cholesterol and fatty acids (Richardson *et al.*, 1996; Gauthier *et al.*, 1999; Izem *et al.*, 2001). The addition of insulin and oleic acid to the growth medium increases CETP synthesis and secretion in SW 872 cells (Richardson *et al.*, 1996). When we grew these cells in the presence of 100 μg/ml fatty acid free BSA/ oleic acid, 1 μg/ml insulin, and 1 μg/ml transferrin, the morphology of the cells changed by 48 hours, the cells appeared enlarged and rounded as opposed to spindle shaped. Staining the cells with oil red O indicated lipid accumulation in these cells (Figure 3.9). The triacylglycerol and cholesterol concentrations increased significantly over a six day time period (Figure 3.10; Figure 3.11). A recent study by Izem *et al.* (2001) found that addition of oleate to the growth medium of SW872 cells caused rapid accumulation of triglyceride-containing droplets as demonstrated by oil red O staining, and also increased the CETP activity and mRNA levels. These observations suggest that the CETP biosynthesis and the cellular lipid pool are tightly linked.

4.3.2 Expression of CETP.CAT gene chimeras in SW 872 cells requires cotransfection of C/EBP$_\alpha$

To understand the molecular mechanisms involved in CETP regulation by fatty acids and cholesterol, we used CETP chimeric gene constructs harbouring sequential deletions of the promoter region linked to CAT as a reporter (150CETP.CAT, 650CETP.CAT, 3200CETP.CAT). These constructs were transfected into differentiated SW 872 cells; however, the expression, as indicated by the CAT activity, was very low (Figure 3.12A). C/EBP$_\alpha$ is required to activate the human CETP promoter by binding to the -250 to –200 bp region (Agellon *et al.*, 1992). Cotransfection of the C/EBP$_\alpha$ expression vector significantly increased the expression of 150CETP.CAT and 650CETP.CAT but had little effect on the expression of 3200CETP.CAT (Figure 3.12B). These results indicate that C/EBP$_\alpha$ enhances the expression of 150CETP.CAT and 650CETP.CAT, whereas 3200CETP.CAT is already expressed to the maximum.

4.3.3 Regulation of the human CETP gene by 25-OH cholesterol is via LXR$_\alpha$/RXR$_\alpha$

The cholesterol mediated induction of the CETP gene is known to involve LXR$_\alpha$/RXR$_\alpha$ and this effect is contained within 400 bp of the promoter region (Luo *et al.*, 2000). These previous studies were carried out using 3T3-L1 cells. To confirm these findings in SW 872 cells, we transfected the CETP.CAT gene chimeras along with expression vectors for LXR$_\alpha$/RXR$_\alpha$ and treated the transfected cells with 25-OH cholesterol. The expression of 650CETP.CAT increased 3.3 fold when 25-OH

cholesterol was added to the growth media; however, there was no effect on the expression of 150CETP.CAT or 3200CETP.CAT (Figure 3.13). These results confirm the previous findings that cholesterol mediated regulation of the CETP gene is contained within 650 bp of the promoter region and requires LXR_α/RXR_α.

4.3.4 Regulation of the human CETP chimeric gene constructs by oleic acid

Fatty acids regulate the gene expression of various enzymes via the transcription factors $PPAR_\alpha/RXR_\alpha$ (Issemann *et al.*, 1990). Oleic acid had no effect on CETP gene regulation when $PPAR_\alpha$ and RXR_α were not coexpressed along with the CETP chimeric gene constructs (Figure 3.14). However, when $PPAR_\alpha/RXR_\alpha$ were coexpressed with CETP chimeric gene constructs, the expression of 650CETP.CAT was significantly increased with no change in the expression of 150CETP.CAT and 3200CETP.CAT (Figure 3.15). This suggests that $PPAR_\alpha/RXR_\alpha$ mediates the regulation of CETP gene by fatty acids and this interaction occurs within the -650 bp region of the CETP gene 5' regulatory region.

Since both oleic acid and cholesterol independently regulate the CETP gene via $PPAR_\alpha/RXR_\alpha$ and LXR_α/RXR_α respectively, we further investigated whether fatty acids and cholesterol interact to regulate CETP gene expression. Treating SW872 cells with 25-OH cholesterol and oleic acid together increased the expression of 3200CETP.CAT with no change in the expression of 150CETP.CAT and 650CETP.CAT when $PPAR_\alpha/RXR_\alpha$ were cotransfected along with CETP.CAT gene chimeras (Figure 3.16). This finding is different from treatment with oleic acid alone that increased the

expression of 650CETP.CAT with no effect on 3200CETP.CAT when CETP.CAT gene chimeras were cotransfected with $PPAR_\alpha/RXR_\alpha$. These results suggest that a) cholesterol interferes with the ability of oleic acid to stimulate CETP expression of 650CETP.CAT, and b) there might be other factors involved located within -650 bp to -3200 bp of the CETP promoter which are activated by both cholesterol and oleic acid.

Treatment of SW 872 cells with both 25-OH cholesterol and oleic acid increased the expression of 650CETP.CAT when coexpressed along with LXR_α and RXR_α to the same extent as observed with 25-OH cholesterol alone (Figure 3.17). This suggests that oleic acid has no effect on cholesterol mediated induction of 650CETP.CAT via LXR_α/RXR_α. On the other hand, giving 25-OH cholesterol and oleic acid together increased the expression of 3200CETP.CAT but 25-OH cholesterol alone had no effect (Figure 3.13). These observations again suggest that there might be other factors involved which interact with the -650 bp to -3200 bp 5' regulatory region of CETP which are activated by both oleic acid and cholesterol.

Different responses of CETP.CAT gene chimeras to oleic acid and cholesterol when transfected with either $PPAR_\alpha/RXR_\alpha$ or LXR_α/RXR_α suggests that LXR_α, RXR_α and $PPAR_\alpha$ might cross talk to regulate CETP gene expression. Oleic acid and cholesterol had no effect on the expression of 650CETP.CAT when cells were cotransfected with LXR_α, $PPAR_\alpha$, and RXR_α (Figure 3.18). There was also no effect seen in 650CETP.CAT expression when oleic acid and cholesterol were added to cells coexpressing $PPAR_\alpha$ and RXR_α. However, when cells are cotransfected with LXR_α/RXR_α, cholesterol and oleic acid increased 650CETP.CAT expression. These

results suggest that the presence of both cholesterol and oleic acid prevents the activation of the respective transcription factors, i.e. LXR_α/RXR_α and $PPAR_\alpha/RXR_\alpha$. The addition of oleic acid to a cholesterol supplemented diet in hamsters prevented a cholesterol mediated increase in CETP activity (Kurushima *et al.*, 1995a) further confirming that oleic acid and cholesterol interfere with each other in regulating CETP, perhaps by disrupting the binding and/or activation of transcription factors.

Previous studies have shown that LXR and PPAR interact *in vitro* (Miyata *et al.*, 1996). LXR_α binds directly to $PPAR_\alpha$ and RXR_α but $LXR_\alpha/PPAR_\alpha$ does not form a DNA binding complex to PPRE or LXR_α response elements (LXRE). LXR_α inhibits binding of $PPAR_\alpha/RXR_\alpha$ to PPRE and antagonizes peroxisome proliferator signaling mediated by $PPAR_\alpha/RXR_\alpha$. On the other hand, $PPAR_\alpha$ inhibits binding of LXR_α/RXR_α to LXRE. Our results in these transfection experiments suggest that there is some kind of interaction between the three transcription factors to regulate the CETP gene expression and this effect depends on the availability of the ligands.

4.3.5 Regulation of the human CETP chimeric gene constructs by stearic acid

The addition of dietary cholesterol to the SFA diet had no significant effect on CETP gene expression in CETP-TG mice (Figure 3.5). However, the regulation of CETP by stearic acid itself is not known. Thus, *in vitro* experiments were performed to investigate whether stearic acid regulates CETP gene expression directly and whether cholesterol interferes with this regulation.

Treatment of transfected SW 872 cells with stearic acid decreased the expression of 650CETP.CAT with no change in the expression of 150CETP.CAT and 3200CETP.CAT, when coexpressed with $PPAR_{\alpha}/RXR_{\alpha}$ (Figure 3.19). This suggests that stearic acid has an opposite effect compared to oleic acid on CETP gene regulation, and that the effect is again contained within the 650 bp region of the CETP 5' regulatory region.

Transfected SW 872 cells coexpressing $PPAR_{\alpha}$ and RXR_{α} treated with 25-OH cholesterol and stearic acid decrease the expression of 650CETP.CAT (Figure 3.20). The decrease in 650CETP.CAT expression also occurred with stearic acid alone suggesting that cholesterol does not interfere with stearic acid mediated down regulation of the CETP gene expression.

The addition of stearic acid abolished the cholesterol mediated increase in 650CETP.CAT expression in transfected SW 872 cells coexpressing $LXR_{\alpha}/RXR_{\alpha}$ (Figure 3.21). These results suggest that stearic acid interferes with cholesterol mediated induction of CETP gene expression. The expression of 3200CETP.CAT decreased when stearic acid and 25-OH cholesterol were added to the culture medium of transfected SW 872 cells coexpressing LXR_{α} and RXR_{α} (Figure 3.21). This decrease was not observed when the transfected cells were treated with 25-OH cholesterol alone. The effect of stearic acid on CETP gene expression is opposite to that of oleic acid. However, the response to fatty acids and cholesterol when added together to the culture medium of transfected cells points towards a possible interaction between fatty acids and cholesterol, and the transcription factors involved.

To investigate whether LXR_α, $PPAR_\alpha$, and RXR_α interact to regulate CETP gene expression, SW 872 cells were transfected with CETP.CAT gene chimeras along with LXR_α, $PPAR_\alpha$, and RXR_α. Stearic acid and 25-OH cholesterol treatment of the transfected cells decreased the expression of 150CETP.CAT and 650CETP.CAT (Figure 3.22). The extent of inhibition was greater compared to the cells coexpressing $PPAR_\alpha/RXR_\alpha$ and treated with stearic acid and cholesterol. It is clear from stearic acid and oleic acid treatment experiments that fatty acids and cholesterol, and the respective transcription factors interact to regulate CETP gene expression.

4.4 Conclusions

The addition of cholesterol to a high fat MUFA diet inhibited CETP activity and hepatic mRNA abundance. However, the addition of cholesterol to a high fat SFA diet enhanced CETP activity but had no significant effect on hepatic CETP mRNA abundance. Oleic acid and stearic acid showed opposite effects in cell culture studies using CETP.CAT chimeric gene constructs indicating that the type of fat has different effects on CETP regulation. Moreover, the addition of cholesterol to various fats showed different results, suggesting that there is an interaction between cholesterol and fatty acids to regulate CETP. Whether this interaction is at the level of transcription factors is not clear from our study. Further investigations need to be carried out to understand how various fatty acids interact with cholesterol in CETP regulation. The understanding of regulation of metabolic pathways by cholesterol together with various fatty acids is crucial as these dietary components are normally consumed simultaneously.

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