Cholesteryl Ester Transfer Protein Modulates Liver Sex Hormone Signaling to Alter Triglyceride Metabolism in Male and Female Transgenic Mice

By

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To my parents, who taught me to work hard, be independent,
and not take myself too seriously.

AND

To my wife, Megan, for your everlasting support.

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ApoA1	Apolipoprotein A-I
ApoA4	Apolipoprotein A-IV
ApoA5	Apolipoprotein A-V
ApoC1	Apoliprotein C-I
ApoC2	Apolipoprotein C-II
ApoC3	Apolipoprotein C-III
ApoE	Apolipoprotein E
$ApoE^{-/-}$	ApoE knockout
AR	Androgen Receptor
ARE	Androgen Response Element
ARF1	ADP Ribosylation Factor 1
AUC	Area Under the Curve
CETP	Cholesteryl Ester Transfer Protein
E_2	17-β-Estradiol
ELITE	Early Versus Late Intervention Trial with Estradiol
ERE	Estrogen Response Element
ERα	Estrogen Receptor α
ERβ	Estrogen Receptor β
FPLC	Fast Performance Liquid Chromatography
GDX	Gonadectomy
Gper1	G-protein coupled Estrogen Receptor 1
GPIHBP1	Glycosylphosphatidylinositol anchored HDL Binding Protein 1
HDL	High-Density Lipoprotein
HERS	Heart and Estrogen/Progestin Replacement Study

HL	Hepatic Lipase
LDL	Low-Density Lipoprotein
LDLR	LDL Receptor
$LDLR^{-/-}$	LDLR knockout
LKO-ERα	Liver Knockout of ERα
LKO-SHP	Liver Knockout of SHP
LPL	Lipoprotein Lipase
LRP1	LDLR Related Protein 1
MTP	Microsomal Triglyceride Transfer Protein
Ovx	Ovariectomy
PCSK9	Protein Convertase Subtilisin/Kexin 9
PDI	Protein Disulfide Isomerase
PEPI	Postmenopausal Estrogen/Progestin Interventions
PPARα	Peroxisome Proliferator Activated Receptor α
SDC1	Syndecan1
SHP	Small Heterodimer Partner
SORT1	Sortilin1
SRB1	Scavenger Receptor B-I
TG	Triglyceride
Veh	Vehicle
VLDL	Very-Low Density Lipoprotein
WHI	Women's Health Initiative
WT	Wild type

Chapter I

1. INTRODUCTION AND LITERATURE REVIEW

Overview

Cardiovascular disease has remained the top cause of death in the United States for nearly 60 years. Great strides have been made in understanding what causes cardiovascular disease. Prior to the 1940's, little was known about what caused cardiovascular disease. In 1948, the Framingham Heart Study began to answer the question as to what increased or decreased a person's risk of cardiovascular disease. The Framingham Heart Study and numerous other studies published since the 1940's and 1950's identified classic risk factors, things that increase risk of cardiovascular disease. These included high blood pressure, smoking, high cholesterol, male sex, etc. Understanding of these risk factors progressed to a more nuanced understanding of High-Density Cholesterol (HDL, "good" cholesterol) reducing risk of cardiovascular disease and Low-Density Lipoprotein (LDL, "bad" cholesterol) increasing risk of cardiovascular disease. Identification of these lipid risk factors led to the hypothesis that risk factor reduction could reduce risk of cardiovascular disease. To test this risk factor reduction hypothesis, drugs were developed to target each of these modifiable risk factors. We now know that quitting smoking and reducing blood pressure can reduce risk of cardiovascular disease. We also know that reducing LDL cholesterol can reduce cardiovascular disease with several classes of drugs.

Despite these advancements, more work needs to be done to continue to reduce the impact of cardiovascular disease. Even with a number of drugs available to treat blood pressure and LDL cholesterol, there is still a very large remaining "residual risk" of cardiovascular disease (1). Furthermore, available drugs that "treat" HDL and triglycerides have not been as successful in

reducing risk of cardiovascular disease. Despite dramatic improvement in reducing cardiovascular disease in men over the last 30 years, reducing cardiovascular disease in women has improved only modestly (2). Although we know that women are protected from cardiovascular disease relative to men, we do not yet have drugs that can target the pathways that protect women from cardiovascular disease. This is largely because pathways contributing to sex differences in risk of cardiovascular disease are not well understood. Understanding how women are protected from cardiovascular disease will lead to the development of new drugs that can potentially target these pathways and reduce the remaining "residual risk" of cardiovascular disease. Further understanding of sex differences in lipoprotein metabolism will foster development of new pathways, and potentially, new therapeutic targets for cardiovascular disease. My thesis work has contributed to understanding sex-differences and triglyceride metabolism, an important risk factor for cardiovascular disease.

Male versus Female Sex as a Unique Perspective to Understand Cardiovascular Disease Risk

In general, women are protected from cardiovascular disease[$] compared to men (3-6). Women have lower risk of cardiovascular disease at any given age (3, 5, 6). Also, women have a 10-year delay in myocardial infarction compared to men (6). Male sex was one of the first risk factors discovered to contribute to cardiovascular disease by the Framingham Study. Additionally, female sex mitigates the impact of obesity on cardiovascular disease risk (7). In addition to changes in absolute risk of cardiovascular disease, women also have improved risk

[$] In general, when I refer to cardiovascular disease, I am referring to atherosclerotic cardiovascular disease as it pertains to risk for coronary heart disease, myocardial infarction, coronary atherosclerosis, acute coronary syndrome, and worsening angina due to atherosclerotic disease. I acknowledge that cardiovascular disease can also refer to stroke, peripheral vascular disease, vavular heart disease, cardiac arrhythmia, congestive heart failure and other diseases pertaining to the cardiovascular system. For the purposes of my thesis, I will use cardiovascular disease and atherosclerotic cardiovascular disease interchangeably.

factors for cardiovascular disease. Compared to men, women have higher HDL, lower LDL, lower Very-Low Density Lipoprotein (VLDL) and lower triglycerides (TGs) (8, 9). In particular, women have much faster clearance of plasma TGs (10-13), which reduces risk of cardiovascular disease. Therefore, understanding sex differences may lead to the discovery of pathways that alleviate risk of cardiovascular disease.

Male Sex Hormones as Mediators of Increased Cardiovascular Disease Risk in Men?

One explanation as to why women are protected from cardiovascular disease may be due to the testosterone hypothesis. Testosterone has been hypothesized to contribute to a man's increased risk of cardiovascular disease because men have higher concentrations of testosterone than women. The hypothesis that high testosterone increases cardiovascular risk in men is controversial for several reasons: Firstly, the majority of cross-sectional studies examining the relationship between testosterone levels and cardiovascular disease support an inverse relationship between testosterone and risk of cardiovascular disease (14-23). Certain studies, however, support a neutral (24-27), positive or J-curve (28) relationship between testosterone and cardiovascular disease. In a meta-analysis of testosterone association with cardiovascular disease, testosterone correlated inversely with cardiovascular disease only when men above age 70 were included in the analysis (29). This suggests that age-related decline in testosterone (17) may be responsible for the inverse relationship between testosterone levels and risk of cardiovascular disease. Secondly, studies of testosterone deprivation clearly show increased risk of cardiovascular disease (30-32). This suggests that low testosterone increases cardiovascular disease risk. Thirdly, studies of testosterone therapy have different effects on risk of cardiovascular disease depending on testosterone status prior to treatment. For example, in hypogonadal men, testosterone replacement reduces risk of cardiovascular disease in men (33, 34). In normal men, testosterone therapy seems to increase risk of cardiovascular disease in randomized controlled trials (35-37).

The unexpected conclusion that testosterone improves risk of cardiovascular disease in men may partly be explained by the impact of low testosterone on risk of metabolic syndrome. Metabolic syndrome is associated with higher risk of cardiovascular disease (38), which may be an important confounder in understanding the cardiovascular disease risk associated with testosterone levels. Reduced testosterone levels are associated with increased fasting glucose, fasting insulin and type 2 diabetes (39-43). Testosterone replacement in men with low testosterone improves insulin sensitivity, reduces glucose and insulin, and reduces risk of type 2 diabetes (44-46). In addition, testosterone replacement in hypogonadal men reduces obesity and improves lean muscle mass, both of which would contribute to reducing risk of type 2 diabetes (44). Thus, the "benefit" of testosterone may be related more to improvements in glucose metabolism and insulin sensitivity than improvements in cardiovascular disease, especially when considering the impact of testosterone in hypogonadal men.

The development of tissue-specific Androgen Receptor (AR) knockout (ARKO) models has allowed for the study of AR signaling in lipid metabolism and atherosclerosis. Mice with a global AR knockout (ARKO) had worsened atherosclerosis relative to controls on an Apolipoprotein E knockout ($ApoE^{-/-}$) background (47, 48). Global ARKO mice had increased weight gain, increased plasma cholesterol and TGs, increased liver TG content and impaired glucose metabolism. Additionally, 5α-dihydrotestosterone, a non-aromatizable AR agonist, reduced atherosclerosis, obesity, plasma cholesterol, and plasma insulin liver TG content and reduced atherosclerosis. These data suggest that AR signaling reduces atherosclerosis and improves glucose and lipid risk factors for cardiovascular disease. While informative on AR function, these mouse models do not recapitulate the elevated risk of cardiovascular disease seen in men. Therefore, better mouse models are needed to recapitulate the effects of AR signaling on risk of cardiovascular disease.

Thus, the testosterone hypothesis is inadequate to explain the increased risk of cardiovascular disease in men compared to women. If anything, testosterone levels in men

inversely correlate with cardiovascular disease risk. This is primarily driven by an age-related decline in testosterone, which also contributes to increased risk of metabolic syndrome. Testosterone may still play an important part in risk of cardiovascular disease in men through effects on signaling and gene expression. Signaling mechanisms regulated by testosterone that contribute to cardiovascular disease risk are not well understood. Studies in mouse models using tissue and global knockout of AR do not model the potential negative effects of testosterone on cardiovascular disease. Further understanding of pathways by which testosterone signaling, independent of testosterone level, may yield insights into why men have increased risk of cardiovascular disease relative to women.

Mechanisms of Testosterone Signaling

Understanding how sex-differences contribute to risk of cardiovascular disease will require an understanding of targets and pathways that are different between males and females and how these targets and pathways contribute to molecular targets involved in the pathogenesis of cardiovascular disease. Testosterone is considered the male sex hormone because it dictates male sexual developments and maintains male sexual function throughout life after puberty. Testosterone can alter cell metabolism through effects on gene transcription through AR and through non-genomic signaling mechanisms. AR is a classic steroid hormone receptor that enters the nucleus after binding of testosterone (49, 50). Once in the nucleus, AR regulates gene transcription by classic hormone-receptor signaling to androgen-response elements (AREs) in promoter and enhancer regions of target genes (49, 50). In addition to classic, ARE-mediated transcription, AR has several non-genomic signaling pathways. Testosterone binding to AR can mediate cell signaling through several pathways. Non-genomic AR signaling can stimulate the Mapk/Erk pathway, the PI3K/Akt/mTor pathway (51). In addition, membrane associated receptors have been shown to mediate some actions of testosterone (51). Membrane-associated AR can mediate signaling via regulation of intracellular calcium and

through regulation of ion channels (51). In addition, other plasma membrane associated receptors, including the sex-hormone binding globulin receptor and EGFR, can mediate effects of testosterone on cell signaling. This modulation of cell signaling by testosterone can not only change intracellular signaling events, but also can influence transcription by both AR and non-AR transcription factors.

Body Fat Distribution as an Explanation for Sex Differences in Cardiovascular Disease Risk?

Another potential hypothesis to explain the sex difference in risk of cardiovascular disease is that differences in body fat distribution alter risk of cardiovascular disease. Women have more subcutaneous fat, whereas men have more visceral fat. Additionally, body fat distribution changes in women from a more subcutaneous distribution to a more visceral distribution of fat with menopause (52). Since body fat distribution, as measured by waist-to-hip ratio, predicts risk of cardiovascular disease (53, 54), women may have lower risk of cardiovascular disease due to a more favorable body fat distribution. The hypothesis that body fat distribution contributes to risk of cardiovascular disease was put forth by Vague in 1947 (55). Experimental evidence to prove this hypothesis would take many decades, but two large prospective studies confirmed that body fat distribution did indeed predict risk of future cardiovascular disease (53, 54). Exercise and weight loss can reduce waist to hip ratio and reduce risk of cardiovascular disease, but long-term weight loss in obese patients remains a clinical challenge due to weight regain. Pharmacologic agents that modify body fat distribution are not currently available. Furthermore, a pooled meta-analysis found that waist-to-hip ratio contributed to cardiovascular risk similarly between men and women (56). Therefore, it is important to understand other factors that may explain how women have lower risk of cardiovascular disease relative to men.

Female Sex Hormones as Mediators of Reduced Cardiovascular Risk in Women?

In addition to the body fat distribution hypothesis and testosterone hypothesis, the estrogen hypothesis suggests that the higher level of estrogen in women protect women against cardiovascular disease. This hypothesis was developed because estrogen is one of the predominant female sex hormones. In support of this, postmenopausal women have increased risk of cardiovascular disease compared to premenopausal women (57-60). A number of prospective studies conducted in the 1970's through the 1990's suggested that estrogen replacement in postmenopausal women improved risk of cardiovascular disease (61-74). Prospective studies, while informative, are subject to various sources of bias. One potential source of bias in prospective studies of estrogen replacement has been labeled the "healthy woman" bias (75), a form of selection bias. This source of bias is due to active seeking of medical care. Women more willing to be on hormone replacement therapy are also more likely to monitor their health and take other medications to treat other diseases, thus enriching for a population that is healthier overall.

To definitively determine the effect of estrogen replacement on risk of cardiovascular disease, a number of randomized controlled trials aimed to experimentally determine whether hormone replacement therapy could prevent cardiovascular disease in postmenopausal women. Two of the largest randomized controlled trials, conducted in 1990's, were The Women's Health Initiative (WHI) and the Heart and Estrogen/Progestin Replacement Study (HERS) (76, 77). Hormone replacement therapy consisted of estrogen and progestin if the women had an intact uterus, or estrogen alone if the women had a prior hysterectomy. The WHI trial enrolled over 16,000 postmenopausal women and monitored cardiovascular disease outcomes over an average of 5.6 years. The HERS trial enrolled over 2,700 women and monitored cardiovascular disease outcomes over an average of 6.8 years (78). Despite the improvement in lipid and diabetes risk factors, hormone replacement therapy did not improve cardiovascular disease in the HERS trial. In the WHI trial, hormone replacement therapy actually worsened cardiovascular disease risk. The increased cardiovascular disease risk associated with hormone replacement

in the WHI was the worst in women who had been assigned to hormone replacement over 10 years after the onset of menopause. This led to the development of the “timing hypothesis”, which suggests that estrogen replacement is most beneficial if initiated soon after menopause, and potentially harmful if initiated late (>10 yr) in menopause.

The Early versus Late Intervention Trial with Estradiol (ELITE) study was designed to test the timing hypothesis (79). The ELITE study enrolled over 600 postmenopausal women and randomized them to treatment with placebo or estrogen. Women in the ELITE trial were stratified into two groups – one group of women were considered in early menopause if menopause occurred in the last 6 years and the other group of women were considered in late menopause if menopause occurred at least 10 years prior to enrollment in the study. Women were followed 5 years and carotid intima medial thickness was used as a measure of atherosclerosis. Estrogen replacement delayed the progression of atherosclerosis in the early menopause group, but failed to delay atherosclerosis in the late menopause group. This result supports timing hypothesis of estrogen replacement. Coronary atherosclerosis was approximated using coronary artery calcium imaging, but this measure was added late to the ELITE trial. Estrogen did not alter coronary atherosclerosis in either the early or late menopause group. It is unclear whether the failure to detect a difference in coronary atherosclerosis was due to insufficient power or the ineffectiveness of estrogen to reduce coronary atherosclerosis. A post-hoc analysis of recently postmenopausal women (age 50-59) in the WHI supported that estrogen replacement reduced coronary atherosclerosis as measured by coronary calcium imaging (80). Further work will be needed to confirm whether estrogen improves cardiovascular disease outcomes in addition to the improvements detected with imaging. Interestingly, estrogen increased plasma TGs in this study, in agreement with the WHI and HERS trial. This may suggest that estrogen-mediated increases in TGs may mitigate other improvements in plasma lipids with regard to risk of coronary heart disease.

To determine the mechanisms of how hormone replacement may reduce risk of cardiovascular disease, a number of studies measured the impact of hormone replacement on risk factors for cardiovascular disease. In one of the first randomized trials of hormone replacement, the Postmenopausal Estrogen/Progestin Interventions (PEPI) study compared the effects of hormone replacement with estrogen and progestin and estrogen replacement on risk factors for cardiovascular disease (81). Hormone replacement reduced total cholesterol, raised HDL cholesterol, reduced LDL cholesterol. Interestingly, much of the effect of hormone replacement was recapitulated in the group treated with estrogen alone (82), suggesting that estrogen is responsible for the improvement in plasma lipids seen with hormone replacement. For the purposes of this introduction, the impact of hormone replacement on risk of cardiovascular disease will focus on estrogen replacement for simplicity. These results on improvements in cholesterol were seen in both the WHI and HERS trials (76, 83, 84). In addition to improvements in cholesterol metabolism, estrogen replacement reduced plasma glucose and insulin levels. This reduced insulin and glucose levels ultimately reduced incidence of impaired glucose tolerance and reduced incidence of type 2 diabetes (85-89). Thus, estrogen improves risk of type 2 diabetes, a negative risk factor for coronary heart disease.

Despite improvements in a number of risk factors, estrogen replacement therapy in postmenopausal women had certain negative effects on risk of cardiovascular disease. Estrogen replacement increased plasma TGs in the WHI, HERS and ELITE trials along with a number of prospective studies of estrogen (76, 81-84, 90-92). Several studies have demonstrated that this increase in plasma TGs is due to increased VLDL production (93-95). Mechanisms responsible for this estrogen-mediated increase in VLDL production are unknown. Furthermore, estrogen replacement is limited by the theoretical increased risk of breast cancer. Further understanding of the mechanisms contributing to estrogen-mediated increases in VLDL-TG may lead to more targeted therapies that improve the effectiveness of estrogen in preventing cardiovascular disease.

While the literature largely supports the hypothesis that estrogen treatment is beneficial in women with regard to cardiovascular disease risk, randomized controlled trials testing the estrogen hypothesis have yielded mixed results. The WHI and HERS trial suggest at best, that estrogen does not reduce risk of cardiovascular disease, and at worst, that estrogen increases risk of cardiovascular disease. The ELITE trial supports the timing hypothesis in that women receiving estrogen soon after menopause have delayed atherosclerosis compared to women receiving estrogen over 10 years after menopause. The majority of lipid and metabolic risk factors improve with estrogen treatment. Estrogen treatment increases plasma TGs by increasing VLDL production, but mechanisms for this are lacking. Since increased plasma TGs are associated with increased risk of cardiovascular disease, the increase in plasma TGs caused by estrogen may mitigate some of the beneficial aspects of estrogen replacement therapy on cardiovascular disease risk. A better understanding of factors that contribute to estrogen-mediated increases in plasma TGs may lead to more targeted therapies reduce risk of cardiovascular disease.

Mechanisms of Estrogen Signaling

Understanding how women are protected from cardiovascular disease relative to men will require an understanding of pathways specific to women that contribute to the pathogenesis of cardiovascular disease. Estrogen, largely considered the female sex hormone along with progesterone, can signal through several estrogen receptors. Estrogen is transported in plasma by sex-hormone binding globulin and passively diffuses across the cell membrane into target tissues. The uterus is thought to be the classic estrogen-responsive target tissue since estrogen increases proliferation of the uterine lining. Thus, uterine mass can serve as a proxy for total body estrogen levels. Many other tissues besides the uterus are sensitive to estrogen action. In a transgenic mouse model designed to detect estrogen signaling, the liver was actually the most

responsive to estrogen (96). Estrogen can mediate its biologic effects through a number of mechanisms.

The classic mechanism of estrogen action involves estrogen binding to the steroid hormone receptors, Estrogen Receptor alpha (ERα) or Estrogen Receptor beta (ERβ). ERα and ERβ have the classic features of steroid hormone receptors – an Activation Function 1 (AF1) domain, a ligand binding domain, a DNA binding domain and an Activation Function 2 (AF2) domain (Figure 1.1A) (97). When unbound to ligand, ERα and ERβ are retained in the cytosol by association to Heat Shock Protein 90 (Hsp90) complexes. Estrogen binding to either ERα or ERβ causes a conformational change that promotes dissociation from Hsp90, dimerization and translocation into the nucleus. Once in the nucleus, ERα and ERβ bind to genomic locations based on sequence recognition of the DNA binding domain (97). These genomic sequences are commonly referred to as Estrogen Response Elements (EREs) and are commonly in the promotor or enhancer regions of genes regulated by estrogen (Figure 1.1B). In addition to binding to genomic location via direct recognition of EREs, ERα and ERβ can bind to genomic locations indirectly, via protein-protein binding via other transcription factors (97). ERα and ERβ can promote or inhibit gene transcription depending on the transcriptional machinery available at a particular genomic location.

In addition to classic effects of estrogen on gene transcription, estrogen can also alter cell signaling via binding to receptors localized to the plasma membrane. ERα and ERβ have been shown to localize to the plasma membrane (Figure 1.1C) (98-100). Membrane localization is achieved through palmitoylation of a serine residue and association with caveolin-1 (Cav-1) (100). Membrane ERα and ERβ signal through the ERK 1/2 and the PI3K pathways (98-100). In addition to membrane localized ERα and ERβ, estrogen can signal through another cell surface receptor, G-protein coupled Estrogen Receptor 1 (Figure 1.1C, Gper1, also called Gpr30) (101). Estrogen binding to Gper1 initiates two signaling cascades – one results in increases in cyclic

AMP (cAMP), the other results in increases in intracellular Ca^{2+} (101). Some of the signaling mediated by Gper1 also involves activation of Epidermal Growth Factor Receptor (EGFR) (101). Although Gper1 is most well characterized for its ability to regulate cell signaling, Gper1-mediated cell signaling may also regulate gene expression since estrogen treatment has been shown to alter gene expression in ERα/ERβ double knockout mice (102). Currently, the relative contribution of membrane signaling from ERα, ERβ or Gper1 to gene expression is not well understood.

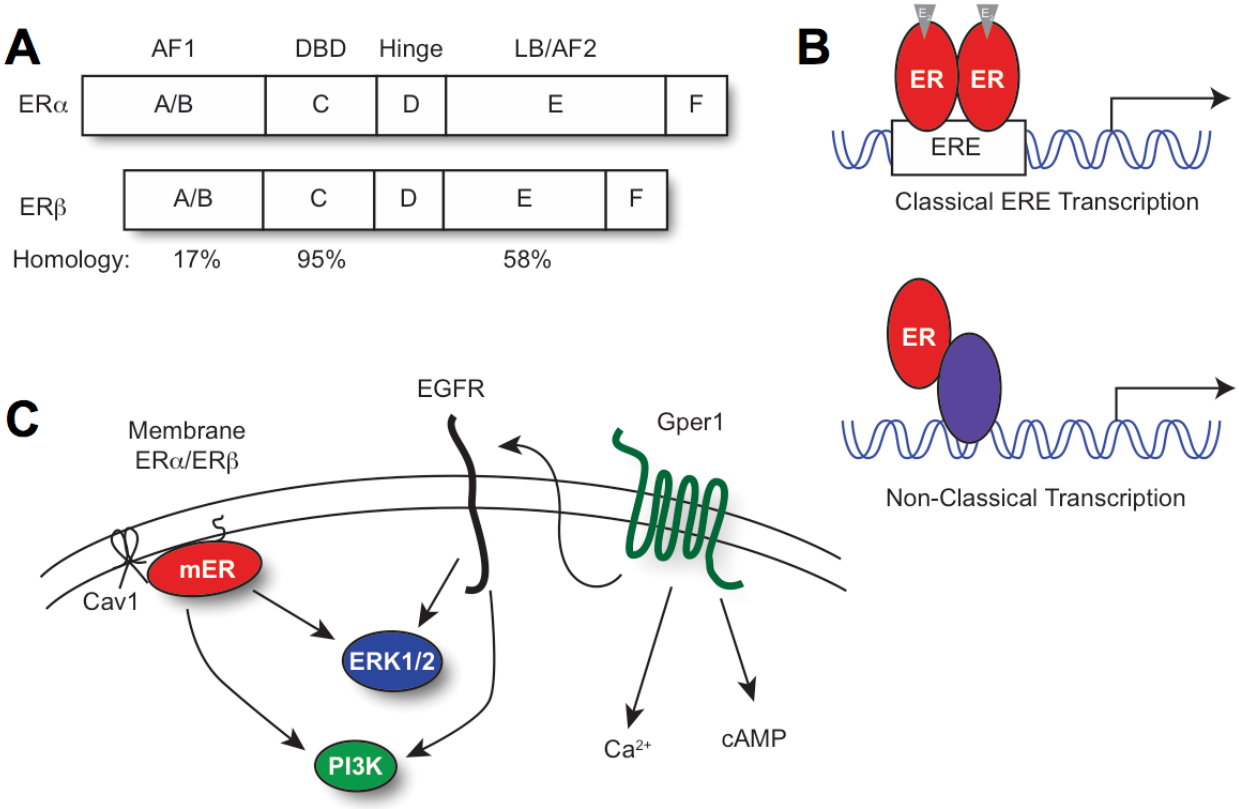

Figure 1.1. Mechanisms of Estrogen Receptor Signaling.
A. Molecular biology structure of Estrogen Receptor α (ERα) and Estrogen Receptor β (ERβ) (Adapted from Marino *et al. Curr Genomics.* 2006). **B.** Classical and Non-Classical ER signaling. **C.** Non-genomic signaling of membrane ER (mER) and G-Protein coupled Estrogen Receptor 1 (Gper1, aka Gpr30).

The Contribution of Estrogen Receptors to Lipid Metabolism and Atherosclerosis in Mice

The advent of mouse genetics has allowed for a mechanistic understanding of how estrogen receptors contribute to various aspects of metabolism. Global, tissue-specific and double knockout mouse models have been developed to study the various contributions of ERα, ERβ, and recently, Gper1 to estrogen function. Due to the diverse role of estrogen and estrogen receptors in a number of signaling pathways and a number of aspects of physiology, this discussion of estrogen receptor function will focus on the role of estrogen receptors in atherosclerosis and lipid metabolism, with a focus on TG metabolism.

ERα is considered to mediate many of the beneficial effects of estrogen on risk factors for cardiovascular disease in mouse models. A number of studies have demonstrated that estrogen reduces atherosclerosis in mice using atherosclerosis prone LDLR knockout ($LDLR^{-/-}$) and ApoE knockout ($ApoE^{-/-}$) mice (103, 104). This corresponded with reduced total cholesterol and, unexpectedly, a reduction in plasma TGs. This reduction in plasma TGs indicates that the effect of estrogen treatment on TG metabolism in mice does not mirror estrogen treatment in humans, in which estrogen actually raises plasma TGs. ERα is required for estrogen treatment to reduce atherosclerosis in ApoE knockout mice (105). Additionally, deletion of ERα worsens obesity, glucose tolerance and insulin sensitivity in mice (106-109).

In addition to studying the global effects of ERα on lipid and glucose metabolism, ERα function in the liver has emerged as an important site of estrogen action on mediating beneficial effects of metabolism. Deletion of liver ERα prevents estrogen-mediated increases in insulin sensitivity (109). Deletion of liver ERα enhances lipid accumulation in liver and prevents estrogen-mediated reductions in liver TG content (109-112). Membrane ERα signaling seems to recapitulate the effects of estrogen on liver lipid metabolism (113). Whether liver ERα is required for estrogen to prevent atherosclerosis in mice remains to be determined.

ERβ does not seem to play a major role in mouse models of atherosclerosis. In mice lacking ERβ , estrogen reduced atherosclerosis in ApoE$^{-/-}$ mice (114). Soy isoflavones, which have estrogenic activity, reduced atherosclerosis in ApoE$^{-/-}$ mice, and ApoE$^{-/-}$ mice lacking ERβ , but not in ApoE$^{-/-}$ mice lacking ERα (115). Similarly, estrogen and soy isoflavones reduce total cholesterol similarly in ApoE$^{-/-}$ as they do in ApoE$^{-/-}$ lacking ERβ (115). ERβ selective agonists have been shown to have certain hypoglycemic effects in mouse models of obesity, but this appears to be due ERβ action to increase insulin secretion from β-cells of the pancreatic islet (116). In male mice treated with ERα or ERβ selective agonists, only ERα agonists reduced liver steatosis, likely because ERβ is not highly expressed in mouse liver (117). Thus, ERβ likely does not play an important role in mouse models of atherosclerosis.

The molecular effects of Gper1 are not well known, but some recent studies have provided evidence that Gper1 may play a role in atherosclerosis and lipid metabolism. Gper1 is expressed in the brain, pancreatic islets, muscle, adipose and liver and expression seems to be localized to endothelium (118-120). Interestingly, Gper1 expression in liver is very low compared to humans (119, 120). Gper1 regulates vascular contractility, smooth muscle cell proliferation, obesity, plasma cholesterol and plasma TG levels (119, 121-123). Gper1 also regulates glucose tolerance in females, likely due to differences in insulin secretion (119). Additionally, knockout of Gper1 worsens atherosclerosis, especially in the absence of ovarian hormones (122). Interestingly, Gper1 knockout mice have higher TG levels than Gper1 expressing mice, suggesting that Gper1 signaling reduces TG levels (123). In humans, however, estrogen increases TG levels, further highlighting that mouse models do not recapitulate human TG metabolism. These results, however, indicate that Gper1 likely plays an important role in estrogen-mediated regulation of risk factors for cardiovascular disease.

Thus, the development of mouse genetic knockout models has advanced the understanding of potential receptor-mediated pathways by which estrogen treatment ameliorates risk of

atherosclerosis. These receptor knockout studies have identified the importance of ERα and Gper1 in glucose and lipid metabolism and risk of atherosclerosis. Importantly, however, mouse models do not model TG metabolism well. If anything, mouse models suggest that estrogen reduces TG levels, but estrogen is known to increase TGs in women. Thus, development of mouse models that recapitulate how estrogen alters TG metabolism are needed to fully understand the role of estrogen in risk of atherosclerosis. Understanding the role of estrogen in TG metabolism is important because TGs are an important risk factor for cardiovascular disease, especially in women. Further understanding how estrogen contributes to increased TG levels may lead to more targeted therapies that reduce risk of cardiovascular disease.

Triglycerides and Cardiovascular Disease

Normal Triglyceride Physiology

TGs are an important contributor to risk of cardiovascular disease. TGs are hydrophobic, energy dense molecules used for energy and energy storage and a number of pathways contribute to maintaining TGs within a very narrow range in the blood. TGs are transported in the blood by lipoproteins. Lipoproteins are large, macromolecular complexes consisting of a hydrophobic core of TGs, cholesterol and cholesteryl esters encased in a shell of phospholipids. Proteins are associated to the surface of lipoproteins. Proteins on the lipoprotein complex serve many diverse functions, including regulating immunity, coagulation, oxidation of plasma lipids, and ligands for binding to cell surface receptors. Understanding lipoprotein metabolism is critical to understanding the contribution of TGs to risk of cardiovascular disease.

Lipoproteins encompass a diverse group of macromolecular complexes, each with different biophysical properties and different biological functions (124, 125). Major classes of lipoproteins were originally named according to their density. HDL consists mainly of a cholesterol-rich, hydrophobic core. HDL is the smallest class of lipoprotein, ranging from 7-13 nm in size, and is

primarily associated with apolipoprotein A1 (ApoA1). HDL is synthesized as a lipid-poor nascent particle consisting mainly of ApoA1. As nascent HDL circulates in the plasma, it acquires cholesterol from peripheral tissues in a process called cholesterol efflux (126). This mature HDL particle then circulates back to the liver, where it is removed from circulation, in a process termed reverse cholesterol transport (126). LDL has a cholesterol-rich hydrophobic core, ranges in size from 21-27 nm in size, and is associated mainly with apolipoprotein B (ApoB). LDL is formed in the blood from the conversion of larger lipoproteins. VLDL is a TG rich lipoprotein, ranging in size from 30-90 nm, and is associated with ApoB or apolipoprotein E (ApoE). VLDL is synthesized in the liver during fasting and transports lipids from the liver to peripheral tissues. Hydrolysis of the lipid core of VLDL alters the lipid composition and size of the particle and ultimately becomes an LDL particle. Chylomicrons are TG-rich lipoproteins made by the small intestine from lipid absorbed from a meal. Chylomicrons are very large, ranging in size from 200-600 nm, and are associated with ApoB and ApoE. Tissue removal of the core lipid contents of chylomicrons results in the formation of smaller chylomicron remnants. These chylomicron remnants are ultimately cleared from circulation by the liver. Thus, TGs are distributed across a range of different kinds of lipoproteins, each with a different function.

Alterations in the metabolism of any of these lipoproteins can contribute to the derangements in plasma TG levels seen in patients with cardiovascular disease. Plasma TG levels are a product of both production and clearance. TG production is regulated by two sources – VLDL TG production by the liver and intestinal chylomicron production by the small intestines after TG absorption from a meal. The rate of TG production by the gut is determined by the rate and efficiency of TG uptake, as well as the TG content of the meal. In the fasted state, VLDL production by the liver is the predominant source of TG (127). The TG within a VLDL particle is derived from fatty acids that circulate to the liver from peripheral tissues, from stored lipid within the liver, or from newly synthesized lipid in the liver (127). VLDL particle synthesis occurs in the endoplasmic reticulum of hepatocytes via a two-step lipidation process

(Figure 1.2A) (128, 129). As ApoB is co-translationally translocated across the endoplasmic reticulum, Microsomal Triglyceride transfer Protein (MTP) acts as a chaperone for apoB by facilitating the transfer of a small amount of TG to the nascent apoB protein (130). Protein Disulfide Isomerase (PDI) is a subunit of MTP that also facilitates TG transfer to this nascent apoB particle (131-133). Once the nascent apoB particle is made, mass transfer of TG to the nascent particle is achieved through the action of ADP Ribosylation Factor 1 (Arf1) (128, 129). After mass transfer of TG to the nascent particle occurs, the mature, TG rich VLDL particle is trafficked out of the endoplasmic reticulum, to the Golgi apparatus and ultimately out of the hepatocyte in a process involving Sortilin 1 (Sort1) (134). Disruptions in the function of any of the targets that assemble VLDL particles can contribute to TG overproduction or accumulation of TG in the liver if TGs are not efficiently exported.

In addition to VLDL synthesis contributing to plasma TG levels, TG clearance is highly regulated process controlling exit of TG from the blood (Figure 1.2B). TG clearance is regulated by cell surface receptors that hydrolyze the TGs in the core of lipoproteins (i.e. lipoprotein lipase, LPL, hepatic lipase, HL) or by cell surface receptors that mediate whole particle uptake (LDL Receptor, LDLR, LDLR-Related Protein 1, LRP1, VLDL receptor, VLDLR) (135-140). TG clearance is regulated by expression level and protein activity of these receptors. A number of secreted proteins that reside on lipoproteins can regulate the lipolytic activity of LPL and HL, resulting in stimulation of activity or facilitating binding of lipoproteins to the receptors. Secreted proteins that stimulate TG clearance include Apolipoprotein C2, ApoC2, Apolipoprotein-AIV, ApoA4, Apolipoprotein AV, ApoA5, and ApoE (141). Secreted proteins that inhibit TG clearance include Apolipoprotein CI, ApoC1, Apolipoprotein CIII, ApoC3, Angpoeitin Like Protein 3, 4 and 8, Angpt3, Angptl4, Angptl8 (141). In addition, TG clearance can also be regulated by expression of the glycoproteins that anchor LPL and HL to the cell surface, such as by Syndecan 1 (Sdc1) or Glycosylphosphatidylinositol anchored HDL Binding Protein 1 (GPIHBP1) (142, 143). Thus, TG clearance involves a number of cell surface receptors and secreted

cofactors that contribute to stimulating or inhibiting TG from lipoproteins in the blood. Disruption in the expression of any of these TG clearance targets can result in changes in the rate TGs are cleared from the blood and may contribute to increasing or decreasing risk of cardiovascular disease.

Thus, an exquisitely complex system balances both production and clearance of TG in the blood to maintain plasma TG levels in a fairly narrow range, between 100-150 mg/dl. Assuming a 20% body fat composition, plasma TGs account for 0.02-0.04% of the total body TG content. Disruption of any of the steps in either VLDL synthesis or TG clearance can lead to an accumulation of plasma TGs. Our model is that small changes in TG flux through any of these pathways can contribute to large changes in plasma TG levels and ultimately, risk of cardiovascular disease. Understanding these pathways, especially sex-differences in these pathways, regulating VLDL synthesis and plasma TG clearance are important because plasma TGs are risk factors for both cardiovascular disease and type 2 diabetes.

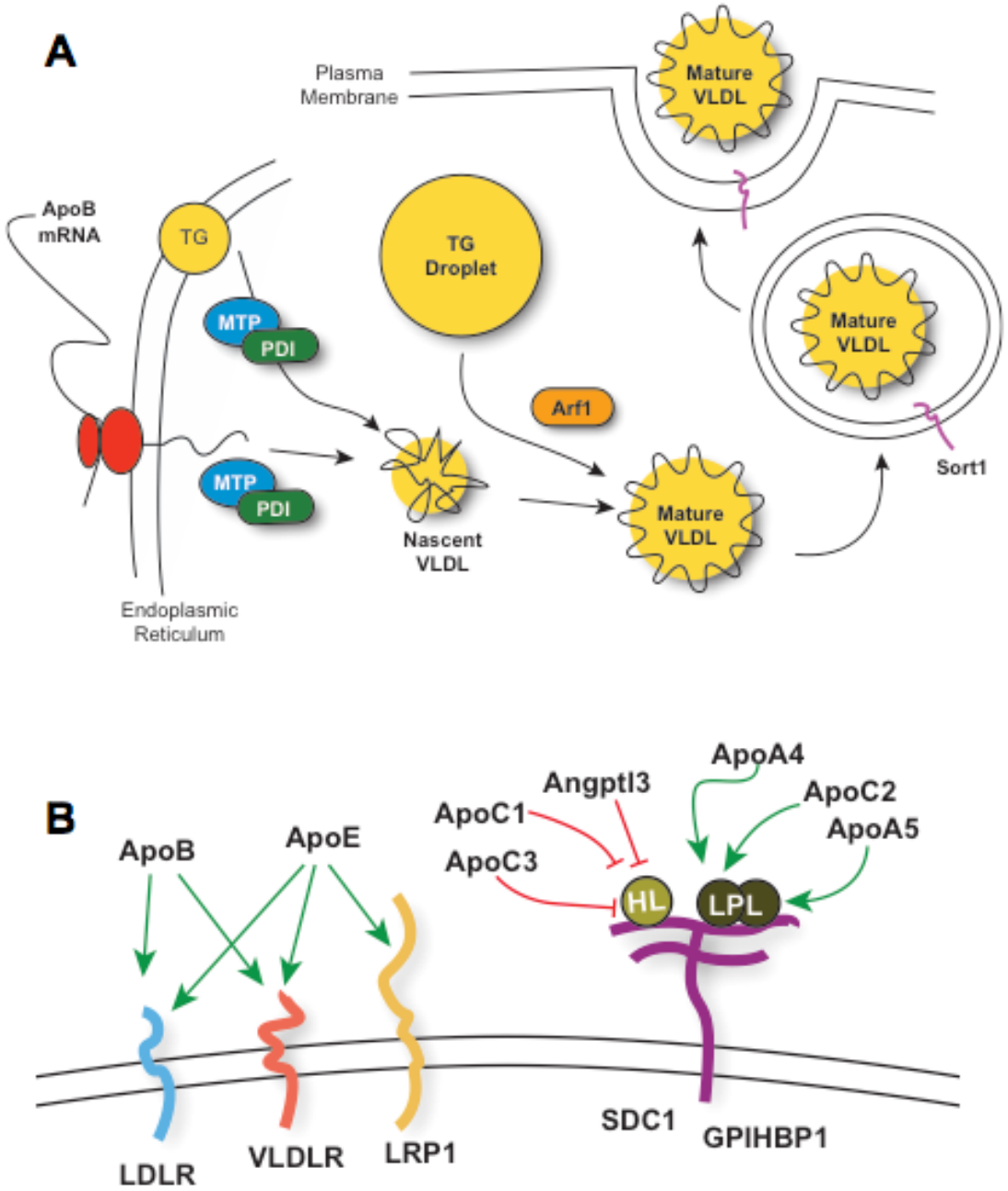

Figure 1.2. Molecular Determinants of VLDL Synthesis and TG Clearance.
A. VLDL Synthesis and Assembly. ApoB mRNA is co-translationally translocated across the endoplasmic reticulum in the hepatocyte. MTP acts as a chaperone for newly synthesized apoB and transfers a small amount of TG onto the nascent apoB protein. PDI is a subunit of MTP and facilitates TG transfer to nascent VLDL. Arf1 transfers of TG from an internal TG droplet to facilitate formation of a mature VLDL particle. Sort1 facilitates vesicular trafficking of mature VLDL particles out of the cell. B. Molecular interactions between secreted proteins regulating TG clearance and cell surface receptors regulating TG uptake. Green arrows indicate stimulation of activity or physical binding. Red arrows indicate inhibition of activity or inhibition of binding. HL and LPL are stimulated or inhibited similarly by secreted proteins.

Plasma Triglycerides are an Underappreciated Risk Factor for Cardiovascular Disease

The first described association of TGs with cardiovascular disease came in the 1950s (144). Additional studies have confirmed the importance of fasting TGs as a risk factor for cardiovascular disease (145-150). In a meta-analysis of plasma TGs and risk of cardiovascular disease, elevated plasma TGs increased risk of cardiovascular disease, but the relationship is not always consistent (151). In women, TGs seem to be a more important risk factor for cardiovascular disease relative to men (148, 149, 151, 152). In addition to the role of fasting plasma TGs in risk of cardiovascular disease, elevated non-fasting plasma TGs increase risk of cardiovascular disease, potentially implicating a causative role for impaired TG clearance in cardiovascular disease (8, 147, 152, 153). Both overproduction of VLDL and impairments of TG clearance contribute to increasing levels of plasma TGs. Studies looking at the role of VLDL production in risk of cardiovascular disease are lacking, due to the difficulty in measuring VLDL production in humans. Studies examining the role of TG clearance are more abundant since TG clearance can be estimated from the change in plasma TG levels over time in response to an oral fat bolus. Studies stratifying patients based on clearance of plasma TGs indicate that impaired TG clearance is an important risk factor for cardiovascular disease (154-159). Thus, elevated fasting TGs, elevated non-fasting TGs and impaired TG clearance contribute to the increased risk of cardiovascular disease, especially in women.

In addition to increasing risk of cardiovascular disease, elevated TGs also increase risk of type 2 diabetes (160, 161). Elevated TGs also increase risk of metabolic syndrome, which is thought to be principally driven by insulin resistance. Insulin resistance contributes to elevated TGs by at least three mechanisms. First, insulin resistance in adipose tissue leads to failure of insulin to suppress lipolysis. This increased lipolysis leads to excess free fatty acid flux to the liver. In the liver, these fatty acids are then re-esterified into TGs and secreted in VLDL. Second, insulin resistance in the liver results in failure to suppress apoB production and MTP activity, leading to VLDL overproduction (162-165). Lastly, impaired TG clearance is also a hallmark of

insulin resistance (166-170). Since type 2 diabetes and metabolic syndrome are associated with increased risk of cardiovascular disease (171, 172), TGs may be a biomarker rather than a causative factor in cardiovascular disease. Nonetheless, in studies examining the impact of elevated TGs on risk of cardiovascular disease, elevated TGs still increased risk of cardiovascular disease in insulin resistant patients or patients with type 2 diabetes, especially in women (173-176). Thus, elevated TGs predict risk of type 2 diabetes, but still predict risk of cardiovascular disease within type 2 diabetic patients.

Human genetic studies have further confirmed the importance of TGs in risk of cardiovascular disease. Mendelian randomization is an approach used to study the causal role of modifiable risk factors on risk of disease (177). Mendelian randomization assumes that certain genetic polymorphisms can account for the modifiable risk factor of interest. The Mendelian randomization approach is less subject to bias than classic epidemiologic approaches, and is considered a “natural” randomized controlled trial. Using certain genetic variants known to influence plasma TGs, Holmes and colleagues used a Mendelian randomization approach on data from 17 studies encompassing over 62,000 individuals to determine the causal role of TGs on risk of cardiovascular disease (178). They identified a set of 27 genetic variants that could account for 0.8% of the variation in plasma TGs without altering either HDL or LDL. Using these variants, they found that plasma TGs indeed increase risk of cardiovascular disease. Interestingly, the authors of this study found a broader group of genetic variants that could account for more of the variation in plasma TGs, but this broader group of genetic variants also reduced HDL and increased LDL cholesterol levels. This may suggest that genetic factors that increase TGs also increase LDL and reduce HDL. Overall, human genetic studies, along with a number of epidemiologic studies, support that elevated TGs increase risk of cardiovascular disease, especially in women.

The Atherogenicity of Triglycerides

Atherosclerosis is the process of the development of fatty lesions in large and small vessels in the body. Atherosclerosis is one of the main process underlying the development of cardiovascular disease. Progression of atherosclerosis causes narrowing of the arteries, leading to the development of atherosclerotic plaques. Rupture of these plaques can trigger an acute clotting cascade that can occlude the affected artery. Occlusion from plaque rupture in the coronary arteries causes a myocardial infarction and occlusion from plaque rupture in the arteries of the brain causes a stroke. Since cholesterol deposits are a hallmark of fatty streaks and advanced plaques in the artery, the causal role of TGs in atherogenesis has been questioned (179). Although the contribution of TGs to atherosclerosis has been debated, TGs may contribute to progression of atherosclerosis by several mechanisms. Lipolysis of TG in VLDL increased expression of inflammatory markers in macrophages (180) and endothelial cells (181). Additionally, macrophage deletion of LPL reduces atherosclerosis in mouse models (182-185). This suggests that lipolysis of TG generates inflammatory signaling molecules that promote macrophage foam cell formation, contributing to progression of atherosclerosis. Perhaps the lack of TG accumulation in atherosclerotic plaques is due to the ability of cells within the atherosclerotic plaque to metabolize TG versus cholesterol. Cholesterol can only be catabolized by conversion to bile acids, which does not readily occur outside of the liver. While TGs are generally considered inert storage molecules within a cell, accumulation of TG can result in excess formation of fatty acids, diglycerides or monoglycerides, which can themselves act as signaling molecules or act as substrates for the formation of other signaling molecules like phospholipids, sphingomyelins, etc. Saturation of the TG storage capacity of a given target tissue will likely cause spillover of TG into these signaling intermediates. Excess flux of TGs can, therefore, serve as a way to stimulate lipid signaling within a target cell or tissue.

Can Pharmacologic Manipulation of TG levels reduce cardiovascular disease risk?

Despite a wealth of studies aimed at understanding the role of TGs and cardiovascular disease risk, only a few clinical trials have attempted to answer whether pharmacologic reduction in TGs can reduce risk of cardiovascular disease. TGs are clearly a risk factor for cardiovascular disease, but whether reduction of TGs by pharmacologic agents improves risk of cardiovascular disease remains to be definitively answered. Statins, niacin, omega-3 fatty acids and fibrate drugs can reduce TG levels in humans. Since statins primarily target LDL cholesterol, niacin primarily targets HDL cholesterol and omega-3 fatty acids target a number of pathways, this discussion will focus on fibrates and the reduction of plasma TGs.

Several fibrate drugs, which target Peroxisome Proliferator Activated Receptor α (PPARα), have been developed to pharmacologically reduce TGs in humans. Clinical trials designed to test the hypothesis that reducing TGs with fibrates reduces risk of cardiovascular disease have been criticized for poor design and for not specifically targeting hypertriglyceridemia (179). Major phase 3 randomized controlled trials have demonstrated that fibrates reduce cardiovascular disease events (186-190), although not with consistent reductions in total cardiovascular mortality (187, 190). Gemfibrozil was shown to reduce cardiovascular disease mortality as a primary preventative measure in asymptomatic men with dyslipidemia (186) or as a secondary preventative measure in patients with low HDL and high TGs (189). Fenofibrate and bezafibrate failed to reduce cardiovascular disease outcomes in type 2 diabetic patients (187, 188) or patients with a previous heart attack with mild dyslipidemia (190). Nonetheless, post-hoc analysis of fibrate trials of patients at high risk for cardiovascular disease demonstrate that reducing TGs reduces risk of cardiovascular disease (189, 191). Additionally, a meta-analysis of 18 fibrate trials reported that fibrates reduce cardiovascular disease events (192). Thus, use of fibrates to lower TGs in high-risk patients, especially those with high triglycerides and low HDL, reduces cardiovascular disease. While fibrates, and perhaps only certain fibrates, can reduce cardiovascular disease in high risk patients, the potency and effectiveness of

available drugs to lower TGs remain relatively poor. Additional therapeutic agents that specifically reduce TGs are needed to determine whether reduction in plasma TGs can reduce cardiovascular disease deaths.

Are Triglycerides an Independent Risk Factor for Cardiovascular Disease?

Despite numerous studies correlating plasma TGs or impaired TG clearance with cardiovascular disease risk, confounding association with HDL has clouded the interpretation that TGs contribute to cardiovascular disease. Elevated plasma TGs are inversely correlated with HDL cholesterol (193, 194). Reduced levels of HDL cholesterol are associated with increased risk of cardiovascular disease (195). Is the increased risk of cardiovascular disease due to elevations in TGs or reductions in HDL level? In an attempt to determine whether TGs are a risk factor for cardiovascular disease independent of HDL levels, several meta-analyses have used multifactorial modeling to control for HDL levels. In one study, adjustment for HDL cholesterol nullified the impact of TGs on cardiovascular disease risk (196). In other studies, however, TGs remained a significant risk factor for cardiovascular disease after adjustment for HDL (8, 151), but this effect remained significant only in women. This has led to the conclusion, by some, that TGs are not an important risk factor for cardiovascular disease. The main factor thought to contribute to the inverse relationship between TGs and HDL levels is Cholesteryl Ester Transfer Protein (CETP) (197, 198).

Cholesteryl Ester Transfer Protein

CETP and Plasma Lipoprotein Metabolism

CETP is a plasma protein that mediates lipid transfer between lipoproteins (Figure 1.3A-B). CETP is expressed by several tissues, including liver, adipose and muscle (199). CETP action results in mass transfer of cholesteryl ester from HDL into LDL and movement of TG out of

apoB containing lipoproteins into HDL (200). Thus, CETP action results in TG accumulation in HDL particles. This TG enrichment of HDL by CETP contributes to reducing HDL levels since TG rich HDL particles are cleared faster from circulation (197, 198). In hypertriglyceridemic patients, CETP mediates mass movement of TG into HDL, contributing to reduced HDL levels (201). Thus, CETP activity explains the inverse association between TG levels and HDL and potentially why adjustment for HDL cholesterol mitigates the statistical impact of TGs on risk of cardiovascular disease. Because of this, pharmaceutical companies developed of CETP inhibitors as a pharmacologic way to raise HDL levels.

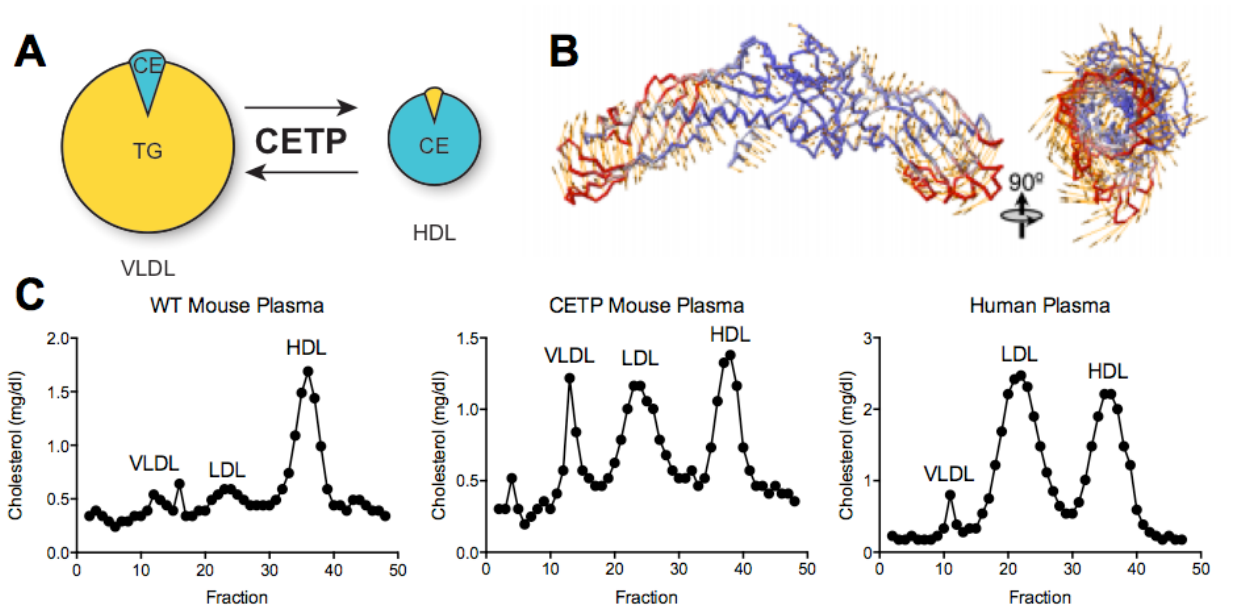

Figure 1.3. General Characteristics of CETP.
A. CETP facilitates lipid transfer between plasma lipoproteins. **B.** Chemical structure of CETP. The hydrophobic core of the CETP molecule facilitates lipid transfer (Image from Zhang *et al. Nat Chem Biol*. 2012). **C.** Mice naturally lack CETP and have cholesterol distributed primarily in HDL (Left Panel). Humans have cholesterol in HDL, LDL and VLDL (Right Panel). Transgenic expression of CETP results in a more human like lipoprotein distribution, with cholesterol in HDL, LDL and VLDL (Center Panel).

CETP and Risk of Cardiovascular Disease

The role of CETP in cardiovascular disease remains controversial despite several decades of research in this area. Several studies have shown that CETP activity is higher in patients with

cardiovascular disease (202-205). Others, however, suggest that CETP is inversely associated with cardiovascular disease (203, 206-211). Human genetic variation in CETP has focused primarily on the genetic variants Taq1B or I405V (212). These variants are associated with small changes in CETP activity. Additionally, promoter variants have been shown to impact CETP activity (212). While certain targeted studies show that variation at certain polymorphisms in CETP associate with cardiovascular disease risk (212-216), whole genome studies fail to demonstrate an association with cardiovascular disease (217-223). This association fails to reach statistical significance, even though genetic variation in CETP is very strongly associated with HDL levels (P<10E-300) (223). Thus, despite analysis of plasma protein activity, mass and genetic variation in many thousands of patients with and without cardiovascular disease, the role of CETP in atherosclerosis is unclear.

Animal Models of CETP and Risk of Cardiovascular Disease

Similar to human studies, animal models of atherosclerosis support both a harmful and protective role for CETP in risk of cardiovascular disease. Similar to humans, study of the role of CETP in cardiovascular disease in animal models supports equivocal roles for CETP in atherosclerosis. Since mice naturally lack CETP, several transgenic models have been developed (224). Transgenic expression of CETP into mice results in a more human like lipoprotein distribution (Figure 1.3C). Certain studies show that CETP expression exacerbates atherosclerosis in mouse models (225-228). Others, however, show that CETP reduces (229-233) or has a neutral effect (234) on atherosclerosis. Rabbits have naturally high levels of CETP and are also susceptible to atherosclerosis using certain genetic strains or using dietary manipulation. Knockdown of expression or inhibition of CETP in rabbit models has been shown to reduce measure of atherosclerosis (235-238). In primates, CETP activity correlated with coronary atherosclerosis (239). Thus, animal models of atherosclerosis have not clarified the role of CETP in atherosclerosis. Mouse models are an important tool in understanding the

mechanisms by which CETP and other targets contribute to risk of cardiovascular disease because the relative ease and efficiency of generating mouse knockout models. Use of genetically manipulated mouse strains facilitates the identification of molecular signaling pathways required for a given phenotype. A key strength of this work was the use of genetic models in the setting of transgenic CETP expression to determine the molecular factors required by CETP to alter aspects of sex hormone signaling and TG metabolism.

CETP Inhibition and Risk of Cardiovascular Disease

Despite the lack of a clear association of CETP with cardiovascular disease, at least five pharmaceutical companies pursued development of drugs aimed at inhibiting CETP as a therapy to reduce cardiovascular disease. Three drugs have completed large scale, phase 3, placebo controlled randomized clinical trials. Torcetrapib was the first CETP inhibitor to be published (240). Torcetrapib raised HDL by 70% in patients at high risk of cardiovascular disease. In spite of this, torcetrapib was associated with increased risk of death from any cause. This was attributed to small increases in blood pressure, which had been a published side effect of torcetrapib prior to pursuit of phase 3 study. Dalcetrapib was the second CETP inhibitor to be published (241). Dalcetrapib was not as potent as torcetrapib in raising HDL (HDL increased 30% in dalcetripib treated patients) and conferred no protection from cardiovascular disease. Evacetrapib was recently halted for inefficacy in 2015 (242). Published results on evacetrapib are not yet available. Anacetrapib and TA-8995 are two CETP inhibitors in clinical development (243, 244). Thus, CETP inhibition has not yet demonstrated a clinical benefit in reducing cardiovascular disease.

CETP inhibition may remain a viable strategy for prevention of cardiovascular disease, not because of HDL elevation, but because of LDL lowering. Recently, anacetrapib has been shown to enhance clearance of apoB containing lipoproteins and reduce TGs (245). Additionally, other CETP inhibitors have been shown to promote clearance of apoB containing lipoproteins (246,

247). If CETP inhibitors reduce LDL in addition to statin treatment, they may reduce cardiovascular disease risk independent of changes in HDL. The mechanism of why inhibition of CETP promotes clearance of apoB containing lipoproteins is currently unknown.

Alternative Functions of CETP

The failure of CETP inhibitors may suggest that CETP has additional functions beyond regulating plasma HDL levels. If CETP has both harmful and beneficial effects on risk of cardiovascular disease, inhibition of CETP may have a neutral effect on cardiovascular disease risk. Several *in-vitro* studies demonstrated that CETP can alter TG metabolism (248-250) . Additionally, CETP has been shown to promote movement of lipid into lipid storage droplets (251). Transgenic expression of CETP in adipose tissue reduces adipocyte size, reduces TG content in adipocytes and alters expression of several genes involved in TG metabolism in adipocytes (252). Additionally, in two meta-analyses of nearly 100,000 patients, genetic variation in CETP was associated with plasma TG levels, in addition to an association with HDL (223, 253). Thus, CETP may have a role in TG metabolism in addition to its well accepted role in HDL metabolism, but studies examining the role of CETP in TG metabolism are lacking.

Studies I worked on in the Stafford laboratory suggest that CETP may contribute to sex-specific effects on metabolism *in-vivo*. Female CETP mice were protected from insulin resistance compared to WT females, whereas male CETP mice were not protected from insulin resistance (254). The protection from insulin resistance in female CETP mice mirrors the protection from type 2 diabetes seen in women compared to men. This suggests that CETP may mediate certain sex-specific effects on metabolism. Genetic variation in *CETP* has been shown to modify the effect of gender on postprandial TG metabolism (255). Additionally, certain genetic variants in *CETP* increase risk of cardiovascular disease in men, whereas the same genetic variants do not impact risk of cardiovascular disease in women (256). Furthermore, sex seems to modify the effect of genetic variation in *CETP* on HDL in several studies (257-259).

This may, therefore, suggest that CETP has sex-specific function in addition to other functions in TG metabolism. Studies of alternative functions of CETP or sex-specific functions of CETP are lacking.

Overview of Thesis Work

An important barrier to development of therapies that target the beneficial effect of female sex on reducing risk of cardiovascular disease is the lack of understanding as to how lipid metabolism differs in females compared to males. Studies of estrogen and estrogen receptors have advanced our mechanistic understanding of some sex differences, but human TG metabolism is not well modeled in rodents. CETP, which mice naturally lack, may be an important contributor to sex differences in lipoprotein metabolism. Understanding the broader role of CETP in lipoprotein metabolism will allow for development of more effective therapies to treat and prevent cardiovascular disease. Since previous work in our laboratory suggested a potential connection between CETP and glucose metabolism in females, I first determined the connection between CETP and estrogen on glucose metabolism. I show that estrogen is required for CETP to alter glucose metabolism. I extend this finding into understanding how CETP contributes to estrogen regulation of TG metabolism. I discovered that CETP contributes both to plasma and TG metabolism in female mice. I further develop the molecular mechanisms required for CETP to alter these aspects of TG metabolism. In extending these studies to males, I found that CETP alters TG metabolism in males, but by a different mechanism than females. Like females, CETP required sex hormones to alter TG metabolism in males. Since CETP is a secreted plasma protein, I reasoned that CETP-mediated changes in lipid flux through a cell-surface receptor may contribute to the impact of CETP on TG metabolism in both males and females. I discovered that LDLR is required for CETP to alter TG metabolism in both males and females, suggesting that LDLR is an upstream determinant of the ability of CETP to alter TG metabolism. This work establishes a novel role for CETP in regulating TG metabolism.

Furthermore, these studies highlight that CETP alters liver sex-hormone signaling to mediate these changes in TG metabolism. Use of the CETP mouse model will be essential for comprehensive understanding of lipoprotein metabolism. Due to the multiple roles of CETP in TG metabolism, development of selective CETP inhibitors may improve the efficacy of CETP inhibitors on reducing risk of cardiovascular disease. A better understanding of how CETP contributes to lipoprotein metabolism will also help the development of therapies for treatment and prevention of cardiovascular disease.

CHAPTER II

2. MATERIALS AND METHODS

Animals

All mouse experiments were approved under the Vanderbilt University Institutional Animal Care and Use Committee. Mice were housed in 12hr light/dark cycles in temperature and humidity controlled facilities with ad-libitum access to chow diet and water. Female mice used in this study were aged 3-5 months old to ensure complete sexual development. All female mice underwent ovariectomy to remove the contribution of endogenous ovarian hormones and reduce variability in natural estrus cycling. After 6-7 days of recovery from ovariectomy, mice were injected subcutaneously with vehicle (sesame oil, S3547, Sigma) or estrogen (1 μg/g, 17-β-estradiol-3-benzoate, E8515, Sigma). Mice were sacrificed 24hr after estrogen treatment. All animals were sacrificed between 8 and 11 am to minimize circadian variation in gene expression.

Transgenic CETP were purchased from the Jackson Laboratories (C57BL/6J-Tg(CETP)UCTP20Pnu/J, Stock No: 001929). Non-transgenic littermates were used as WT controls. CETP mice were bred with $ER\alpha^{flox/flox}$ mice with Cre recombinase under control of the albumin promoter (LKO-ERα, $ER\alpha^{flox/flox}$;ALB-$Cre^{+/-}$, described previously (109)) to generate LKO-ERα CETP ($ER\alpha^{flox/flox}$;ALB-$Cre^{+/-}$;$CETP^{+/-}$) and LKO-ERα littermates. CETP mice were bred with $SHP^{flox/flox}$ mice with Cre recombinase under control of the albumin promoter (LKO-SHP, $SHP^{flox/flox}$;ALB-$Cre^{+/-}$, described previously (260)) to generate LKO-SHP CETP ($SHP^{flox/flox}$;ALB-$Cre^{+/-}$;$CETP^{+/-}$) and LKO-SHP littermates. Mice lacking LDLR were purchased from the Jackson Laboratories (B6.129S7-$Ldlr^{tm1Her}$/J, Stock No: 002207, $LDLR^{-/-}$). CETP mice

were bred with LDLR$^{-/-}$ to generate LDLR$^{-/-}$ and LDLR$^{-/-}$CETP littermates. All strains were backcrossed onto the C57BL/6 background at least 10 generations.

Genotyping

Mice were genotyped using tail DNA. The CETP transgene was detected in a multiplexed PCR reaction containing CETP-F (GAATGTCTCAGAGGACCTCCC), CETP-R (CTTGAACTCGTCTCCCATCAG), Control-F (CTAGGCCACAGAATTGAAAGATCT), Control-R (GTAGGTGGAAATTCTAGCATCATCC). Detection of the ERα^{flox} allele was done using primers ERα-P1 (TTGCCCGATAACAATAACAT) and ERα-P3 (GGCATTACCACTTCTCCTGGGAGTCT). Once the ERα^{flox} allele was bred to homozygosity, only detection of CETP and Cre recombinase were done for genotyping. Detection of the SHP^{flox} allele was done using a multiplex reaction with primers SHP-F (GCCTTTAACTCAAGTACTAGGGAGGCAG), SHP-R1 (CTACCCAGAGCGACATGGTGAGAC), and SHP-R2 (AGTTGTGTCTGGTTCCTGACCTTGG). Once the SHP^{flox} allele was bred to homozygosity, only detection of CETP and Cre recombinase were done for genotyping. Detection of Cre recombinase was done using primers Cre-F (GAACCTGATGGACATGTTCAGG) and Cre-R (AGTGCGTTCGAACGCTAGAGCCTGT) in a multiplex reaction with control primers Myogenin-F (TTACGTCCATCGTGGACAGC) and Myogenin-R (TGGGCTGGGTGTTAGCCTTA). Detection of the LDLR knockout allele was done using a multiplex reaction with primers LDLR-F (CCATATGCATCCCCAGTCTT), LDLR-R1 (GCGATGGATACACTCACTGC), LDLR-Neo-R2 (AATCCATCTTGTTCAATGGCCGATC). Once the LDLR was bred to homozygosity with CETP mice, only CETP was used for genotyping.

Ovariectomy Surgery

Animals were anesthetized under inhaled isoflurane (1-5% diluted in 100% oxygen, 1-2 L/min). Following midline dorsal skin incision, two lateral incisions of the dorsal peritoneal wall

were used to remove each ovary. Single simple interrupted stiches with 5-0 pga suture (034896, Henry Schein) was used to close peritoneal incisions and 9mm autoclips were used to close the skin incision. Ketofen analgesic was injected once preoperatively and every 24 hr. postoperatively for 2 days (100 μl, 5-10 mg/kg, subcutaneous). Ceftriaxone was administered once immediately after surgery (100 μl, 20-40 mg/kg, intraperitoneal). Mice were housed individually following surgery and allowed to recover for 6-7 days prior to study. Mice weighing less than 90% of their pre-surgical mass were not used for study.

In-vivo Hyperglycemic Clamp Testing

C57Bl/6 female and CETP female mice were subject to sham surgery (Sham), ovariectomy (Ovx) or ovariectomy with estrogen replacement (Ovx+E2). Estrogen replacement was accomplished with subcutaneous implantation of continuous release pellets (0.36 mg, 60-day release, Innovative Research of America, Sarasota, FL). Following surgery mice were placed on high fat diet (60% kcal from fat, Research Diets) for 5 weeks. Five days prior to study, catheters were surgically implanted in the carotid artery and jugular vein for intravenous infusion of glucose and arterial blood sampling as described previously (261). On the day of study, mice were fasted 5 hrs. Glucose (50% dextrose) was infused continuously at a variable rate to maintain hyperglycemia (target blood glucose=275 mg/dl). Blood glucose was measured every 5 minutes in the first 20 minutes of the clamp and then every 10 minutes for the remainder of the 2 hr clamp study using a handheld glucometer. Glucose infusion rate was normalized to total body weight.

Lipid and Lipoprotein Analysis

Blood was collected in EDTA-containing tubes (16.444.100, Sarstedt). Plasma TG and cholesterol were measured using colorimetric kits (TR22421, TR13421, Infinity). Plasma

lipoproteins were separated using fast-performance liquid chromatography (FPLC) on a Superose6 column (17-5172-01, GE Healthcare). Liver TG and total cholesterol content was determined by the Vanderbilt Hormone Assay Core. Plasma estradiol was measured by colorimetric ELISA (ES180S-100, Calbiotech). Plasma β-hydroxybutyrate was measured following 18hr fasting and 5hr refeeding using a colorimetric kit (700190, Cayman Chemical). Liver Protein Disulfide Isomerase (PDI) activity was measured (ENZ-51024, EnzoLife Sciences) from liver homogenates made in RIPA buffer supplemented with protease and phosphatase inhibitors (89901, 78440, ThermoFisher).

In-vivo TG Clearance and Production

To measure TG clearance, 12hr fasted mice were orally gavaged with olive oil (200 μl/mouse) and plasma TG was measured from tail blood sampling over 5hr. To measure chylomicron TG production, 12hr fasted mice were given intraperitoneal Triton WR-1339 (500 mg/kg) 30 min prior to oral gavage with olive oil (200 μl/mouse). Plasma TG levels were measured from tail blood sampling over 4hr. To measure TG production, 3hr fasted mice were given intravenous administration of Triton WR-1339 (500 mg/kg, T0307, Sigma) and plasma TG was measured over 2hr.

Liver mRNA Expression

Total RNA was isolated using Trizol (15596018, ThermoFisher) from tissues preserved in RNA-Later (AM7021, Ambion). RNA was treated with Turbo-DNase (Ambion) and cDNA was synthesized by reverse-transcriptase (170-8891, iScript, Bio-Rad). Real-Time RT-PCR with Sybr Green (S5193, Sigma) was used to quantify mRNA expression from 10ng cDNA in triplicate using primers validated for specificity by melting-curve analysis. Gene expression was quantified using the efficiency corrected Pfaffl method (262). Efficiency for each gene was

determined from background subtracted amplification curves using the program LinRegPCR (http://linregpcr.nl) (263). All samples were normalized to the house-keeping gene cyclophilin A (*Ppia*).

Liver Immunoblot and Protein Activity

Liver tissue was stored at -80°C until use. Liver homogenates were made in RIPA buffer (89901, ThermoFisher) with protease inhibitors (2X concentration, 78440, ThermoFisher) and phosphatase inhibitor cocktails 2 and 3 (P5726, P0044, Sigma). Following bead homogenization (zirconium oxide beads, 1-2 min, Setting 8, NextAdvance), liver protein concentration was measured (23225, Pierce). Liver protein was diluted in molecular biology grade water and LDS buffer (B0007, ThermoFisher) and denatured 10 minutes at 70°C. For protein immunoblotting, 20 μg protein was separated on 4-12% Bis-Tris SDS-PAGE gels (NW04127BOX, ThermoFisher) using MES running buffer and transferred to nitrocellulose and incubated in blocking buffer (927-40000, Licor). Following overnight incubation in primary antibody, washing in TBS-T (0.05% Tween 20, P9416, Sigma), and incubation in secondary antibody, immunoblots were visualized using the Odyssey infrared scanner (Licor). Activity of microsomal triglyceride transfer protein (MTP) was determined by a fluorescence kit from liver homogenates prepared as for western blotting (MAK110, Sigma). Briefly, MTP activity was assessed by determining the fluorescence transfer from a donor particle to an acceptor particle using liver homogenates as the source of MTP. Liver homogenates, fluorescently labeled donor particles and acceptor particles were incubated in a black 96-well plate for 24hr at room temperature in light protected conditions. Fluorescence transfer was assessed using a fluorimeter (λex = 465/λem = 535 nm) and MTP activity was expressed as pmol transferred/24hr/μg protein in 10 μl. Activity of protein disulfide isomerase (PDI) was determined by a fluorescent kit (ENZ-51024, Enzo Life Sciences) from liver homogenates prepared as for western blotting. Briefly, PDI activity was assessed

using an insulin aggregation assay with liver homogenates as the source of PDI in the presence of DTT. PDI activity unlinks the disulfide bridges present in the insulin, which does not get reformed due to the presence of DTT. This leads to aggregation of the insulin molecule, which is assessed using a proprietary fluorescent aggregation dye (PDI detection reagent). Liver homogenates (10 μl) were incubated in the presence of insulin (160 μM) and DTT (1 mM). Following a 30-minute incubation at room temperature in light protected conditions, 10 μl of stop reagent and 10 μl PDI detection reagent were added to each well and incubated another 15 minutes at room temperature. Fluorescence was detected using a fluorimeter (λex = 500/λem = 603 nm), and PDI activity was expressed as RFU/hr/μg protein. Western blotting for ApoB (Lifespan Biosciences). Antibodies for ApoE (Meridian Life Science), ApoA1 (Meridian Life Science), ApoC1 (SantaCruz Biotechnology), ApoC2 (SantaCruz Biotechnology), ApoA5 (ThermoFisher), LDLR (Abcam), Pcsk9 (Abcam), SRB1 (Novus Biologicals), LRP1 (Abcam), Sdc1 (SantaCruz Biotechnology), Gpihbp1 (SantaCruz Biotechnology), VLDLR (Abcam), CD36 (Novus Biologicals) and β-Actin (BioRad) were done at a 1/1000 concentration overnight at 4°C.

Statistical Analysis

All data are summarized using mean ± SEM. Statistical tests between two groups were analyzed by unpaired Student's t-test. Data with more than one group were analyzed by 1-way ANOVA with Bonferroni post-hoc comparisons of selected columns. Repeated measures 1-way ANOVA was used for measures of plasma TG over time with Bonferroni post-test comparisons. Genotype effects were determined by 2-way ANOVA. P-values <0.05 were considered statistically significant.

CHAPTER III

CHOLESTERYL ESTER TRANSFER PROTEIN IS REQUIRED FOR ESTROGEN TO ALTER TRIGLYCERIDE METABOLISM IN FEMALE MICE.

(Adapted from Palmisano *et al. J Lipid Res.* 2016 (264))

Abstract

Elevated plasma triglycerides (TGs) increase risk of cardiovascular disease in women. Estrogen treatment raises plasma TGs in women, but molecular mechanisms remain poorly understood. Here we explore the role of Cholesteryl Ester Transfer Protein (CETP) in the regulation of triglyceride metabolism in female mice, which naturally lack CETP. In transgenic CETP females, acute estrogen treatment raised plasma TGs 50%, but not in non-transgenic littermate females. This increase in plasma TGs in response to estrogen was due to increased TG production and increased expression and activity of genes involved in Very-Low Density Lipoprotein (VLDL) synthesis and assembly. We also examined whether CETP expression had effects on TG metabolism independent of estrogen treatment. CETP increased plasma metabolites of liver β-oxidation, increased expression of liver genes involved in β-oxidation, and reduced liver TG content by 60% relative to non-transgenic littermate females. Thus, CETP is required for the hypertriglyceridemic effects of estrogen in female mice. These findings demonstrate a novel role for CETP in estrogen-mediated increases in TG production and a broader role for CETP in TG metabolism.

Introduction

Elevated plasma triglycerides (TGs) are a major risk factor for cardiovascular disease in women (8, 265). Incremental increases in plasma TGs elevate risk of myocardial infarction in women even after multifactorial adjustment for other risk factors, whereas the association between TGs and myocardial infarction is lost after multifactorial adjustment in men (8). Furthermore, estrogen replacement in postmenopausal women raises plasma TGs (84). This increase in plasma TG with estrogen replacement may counteract beneficial effects of estrogen, such as increased insulin sensitivity (266, 267), reduced low-density lipoprotein (LDL) cholesterol, and increased high-density lipoprotein (HDL) cholesterol (84). Several studies demonstrated that estrogen increases very-low density lipoprotein (VLDL) TG production in women (93-95), but the mechanisms behind this remain unknown.

In the fasting state, TGs are packaged into VLDL particles by the liver (268). In the fed state, intestinally absorbed TGs are packaged into chylomicrons (265). Both overproduction of VLDL and delayed clearance of chylomicrons can increase TG levels and increase risk of cardiovascular disease (265, 268). Once lipoproteins enter circulation, tissue lipases and transfer proteins, like Cholesteryl Ester Transfer Protein (CETP), modify the size and lipid content of lipoproteins. CETP facilitates lipid exchange between lipoproteins, resulting in TG enrichment of HDL (199). This CETP-mediated TG enrichment of HDL decreases HDL levels through increased HDL clearance (199, 269). Although CETP inhibitors were developed to raise HDL, CETP inhibitors have not reduced cardiovascular disease risk (240, 241). This may suggest that CETP has additional functions beyond regulation of HDL cholesterol levels. Currently, the role of CETP in regulating liver and plasma TG metabolism is unknown.

In this report, we show that transgenic expression of CETP in female mice is required for estrogen-mediated increases in TG production. Although mice naturally lack CETP, transgenic expression of CETP results in a human-like lipoprotein distribution (270). Previously, CETP was

shown to improve HDL function in women, but not men (271). Additionally, we have shown that transgenic expression of CETP protected against insulin resistance in females (254), recapitulating how estrogen increases insulin sensitivity in women. This suggests that CETP may facilitate estrogen-specific functions. Here, we show that CETP expression also facilitates estrogen action on TG metabolism. Transgenic expression of CETP in female mice results in both estrogen-mediated increases in VLDL production and reduced liver TG content. We demonstrate that increased VLDL production corresponds to increases in Protein Disulfide Isomerase (PDI) mRNA expression and activity in the liver. Additionally, we demonstrate that CETP-mediated reductions in liver TG content correspond with increases in liver mRNA expression of β-oxidation target genes and with plasma ketone levels.

Results

CETP facilitates estrogen-mediated improvements in glucose tolerance.

Previous work demonstrated that expression of CETP in females improved insulin sensitivity and that CETP expression in males had no effect on insulin sensitivity (254), but the mechanism of this female specificity on glucose metabolism is unknown. We hypothesized that CETP facilitates a response to estrogen to mediate effects on glucose metabolism. Estrogen improves glucose metabolism in women (85), but the role of CETP in mediating estrogen's action on glucose metabolism is currently unknown. To determine whether CETP could facilitate the effects of estrogen on glucose tolerance, wild-type (WT) and CETP females were sham treated (Sham), ovariectomized (Ovx) or given estrogen replacement after ovariectomy (Ovx+E2). To assess glucose tolerance, glucose was continuously infused to maintain hyperglycemia (target glucose at 275 mg/dl, Figure 3.1A). Hyperglycemia was used to study glucose tolerance to get glucose levels high enough to alter liver glucose transport (272). Liver glucose metabolism is relevant to CETP mice since the putative mechanism of increased insulin sensitivity in females was due to liver bile acid metabolism (254). In sham treated mice, both WT and CETP mice had very high glucose tolerance (glucose infusion rate >75 mg/kg/min), but CETP females had slightly lower glucose tolerance than WT females (Figure 3.1B). Ovariectomy impaired glucose tolerance similarly in both WT and CETP females (Figure 3.1C). Estrogen replacement restored glucose tolerance in CETP females to levels comparable to sham CETP females (Figure 3.1D). Estrogen treatment did not restore glucose tolerance in WT and may have slightly worsened glucose tolerance compared to ovariectomized WT females (Figure 3.1D). Thus, CETP is required for estrogen to enhance glucose tolerance in the setting of hyperglycemia. These results establish a connection between estrogen action and CETP in glucose metabolism, but the connection between estrogen and CETP on other aspects of metabolism are unknown.

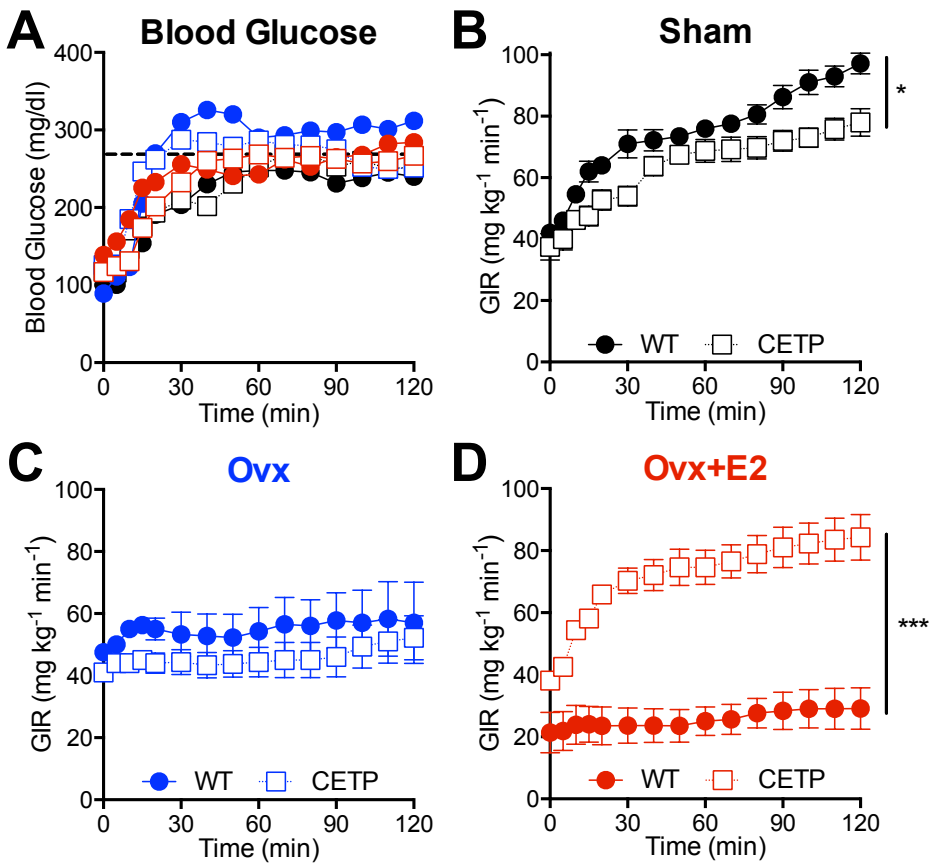

Figure 3.1. Estrogen treatment restores glucose tolerance during hyperglycemia in CETP females.
Wild type (WT) and CETP transgenic mice were maintained on 60% high-fat diet for 5 weeks following sham surgery (Sham), ovariectomy (Ovx), or ovariectomy with estrogen replacement (Ovx+E2, 0.32mg Estrogen, 60-day release, Innovative Research of America). Five days prior to surgery, catheters were surgically implanted in the carotid artery and jugular vein for assessment of glucose tolerance by hyperglycemic clamp. Glucose was infused as needed to maintain hyperglycemia at a target glucose of 275 mg/dl over 120 min. **A.** Blood glucose levels over the course of the hyperglycemic clamp. **B.** Glucose infusion rate (GIR) for sham WT and CETP females. **C.** GIR for ovariectomized WT and CETP females. **D.** GIR for WT and CETP mice undergoing estrogen replacement after ovariectomy (Ovx+E2). *P<0.05, ***P<0.001 (2-Way ANOVA, n=4-9/group). All data are summarized using mean ± SEM.

Estrogen raises plasma triglycerides in CETP females but not WT females.

Since we demonstrated that estrogen required CETP to enhance glucose tolerance, we next tested whether CETP was required to mediate the hypertriglyceridemic effect of estrogen. To determine if CETP was required for estrogen to alter plasma TG metabolism, mice were ovariectomized and given vehicle (sesame oil) or estrogen (1 μg/g body weight, 17β-estradiol-3-benzoate) and euthanized 24hr later. All mice were ovariectomized to remove the contribution of endogenous hormones and to reduce variability in estrus cycling. Estrogen treatment raised plasma estrogen concentration and uterine weight equally in both WT and CETP females after ovariectomy (OVX) (Figure 3.2A-B). Estrogen treatment increased plasma TGs by 50% in CETP mice (55.2±4.9 *vs*. 83.6±6.1 mg/dl, $P<0.01$, Figure 3.2C), but did not alter plasma TGs in WT mice (55.1±4.2 *vs*. 61.9±6.7 mg/dl, Figure 3.2C). Estrogen treatment modestly, but non-significantly, increased plasma cholesterol in CETP females (Figure 3.2D). In CETP mice, estrogen treatment enriched the TG content of VLDL as measured by FPLC (Figure 3.2E-F). The increase in cholesterol in CETP mice treated with estrogen was distributed in VLDL, LDL and HDL (Figure 3.2G-H). VLDL apolipoprotein B (apoB) levels were significantly higher in estrogen treated CETP mice relative to WT mice, suggesting a higher number of VLDL particles after estrogen treatment in CETP mice (Figure 3.2I). These data suggest that CETP is required for estrogen to raise plasma TGs in VLDL.

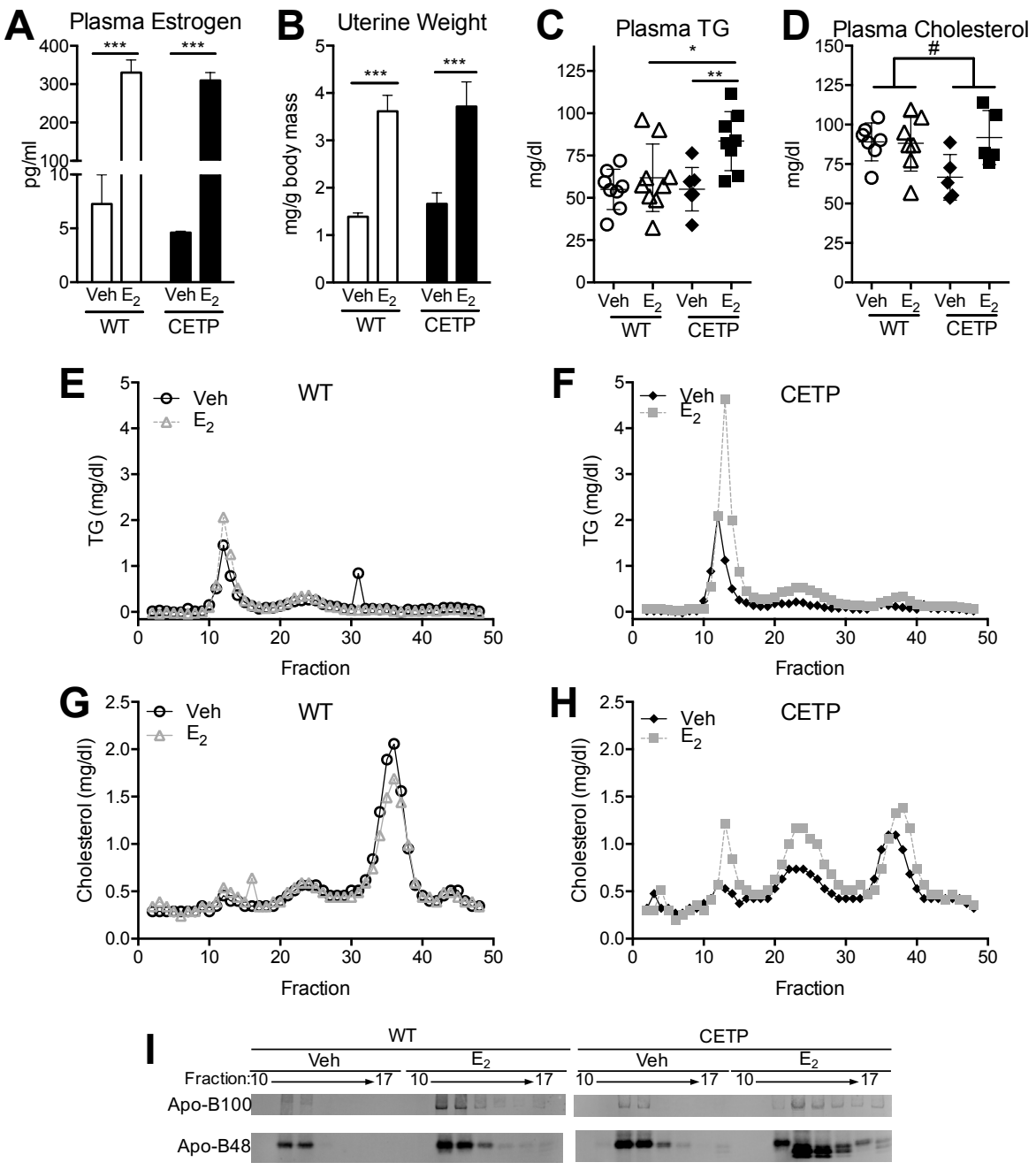

Figure 3.2 Estrogen raises plasma triglycerides in CETP but not WT female mice.
A-D. Ovariectomized female mice with transgenic expression of Cholesteryl Ester Transfer Protein (CETP) and non-transgenic, wild type (WT) littermates were given vehicle (Veh, sesame oil) or estrogen (E_2, 1 μg/g, β-estradiol-3-benzoate) and sacrificed 24hr later. Plasma estrogen levels (A), uterine weight (B), plasma triglyceride (TG) (C), plasma cholesterol (D) in WT and CETP mice given Veh or E2. *P<0.05, **P<0.01, ***P<0.001 (ANOVA, n=7-9/group). #P<0.05 for genotype (2-way ANOVA). E-H. Fast performance liquid chromatography (FPLC) distribution of pooled plasma TG (E-F) and cholesterol (G-H) of WT (E, G) and CETP (F, H) females. I. Western blot for apolipoprotein B of VLDL (FPLC fractions 10-17). All data are summarized using mean ± SEM.

Estrogen treatment raises TG production in CETP females.

To determine how estrogen raises plasma TGs and VLDL-TG in CETP females, we measured plasma clearance and production of TG. Estrogen treatment did not alter postprandial plasma TG concentration after an oral olive oil bolus in either WT or CETP females (Figure 3.3A-B). Since estrogen treatment did not significantly alter postprandial TG concentrations, vehicle and estrogen treated data were pooled within each genotype. CETP expression resulted in a greater postprandial TG excursion relative to WT females (1397.0±157.5 *vs.* 1029.0±61.3 $mg*dl^{-1}hr$, $P<0.05$, Figure 3.3C). Since postprandial plasma TG concentration is a balance of intestinal production of chylomicron TGs and clearance from plasma, we measured chylomicron TG production in vehicle and estrogen treated WT and CETP female mice. Neither estrogen treatment nor CETP expression significantly altered chylomicron TG production (Figure 3.4), indicating the increased postprandial TG excursion in CETP mice is likely due to impaired TG clearance. TG production was measured in fasted mice after administration of the lipoprotein lipase inhibitor Triton WR-1339. In WT females, estrogen treatment modestly, but non-significantly, lowered TG production (Figure 3.3D). In CETP females, however, estrogen treatment raised TG production (Figure 3.3E). TG production was markedly lower in vehicle treated CETP females relative to vehicle treated WT females (179 ± 107.8 *vs*. 360.1 ± 94.71 μmol/kg/hr, CETP veh *vs*. WT veh. Figure 3.3D-E, $P<0.01$). Plasma free fatty acid levels were not different between WT and CETP females regardless of estrogen treatment (Figure 3.3F). Thus, plasma TGs were not different between vehicle treated CETP and WT females due to the net effect of reduced VLDL-TG production and delayed TG clearance in CETP females. Estrogen treatment, however, raised plasma TGs through enhanced TG production in CETP females but not in WT females.

Since VLDL production by the liver is the main source of TGs in the fasted state, we sought to understand if estrogen treatment altered expression of genes of VLDL synthesis and

assembly in WT and CETP mice (for review of VLDL assembly (128)). Liver mRNA expression of apoB (encoded by *Apob)* and Microsomal Triglyceride-transfer Protein (MTP, encoded by *Mttp)* were increased in CETP females relative to WT, but did not change with estrogen treatment (Figure 3.3G). Liver MTP activity was lower in CETP females relative to WT, but did not significantly change with estrogen treatment (Figure 3.5). Protein Disulfide Isomerase (PDI, encoded by *P4hb, Pdia3, Pdia4*), is a critical subunit of MTP (130). Overexpression of PDI is sufficient to facilitate TG export even when MTP levels are low (132). Estrogen increased expression of several isoforms of PDI (*P4hb, Pdia3, Pdia4*) in CETP females, but not in WT females (Figure 3.3G). Corresponding with increased mRNA expression of PDI with estrogen treatment, liver PDI activity increased 4-fold with estrogen treatment in CETP females, but not in WT females (Figure 3.3H). Taken together, these data indicate that CETP raises plasma TGs with estrogen treatment by increasing VLDL-TG production and increasing expression and activity of PDI in the liver.

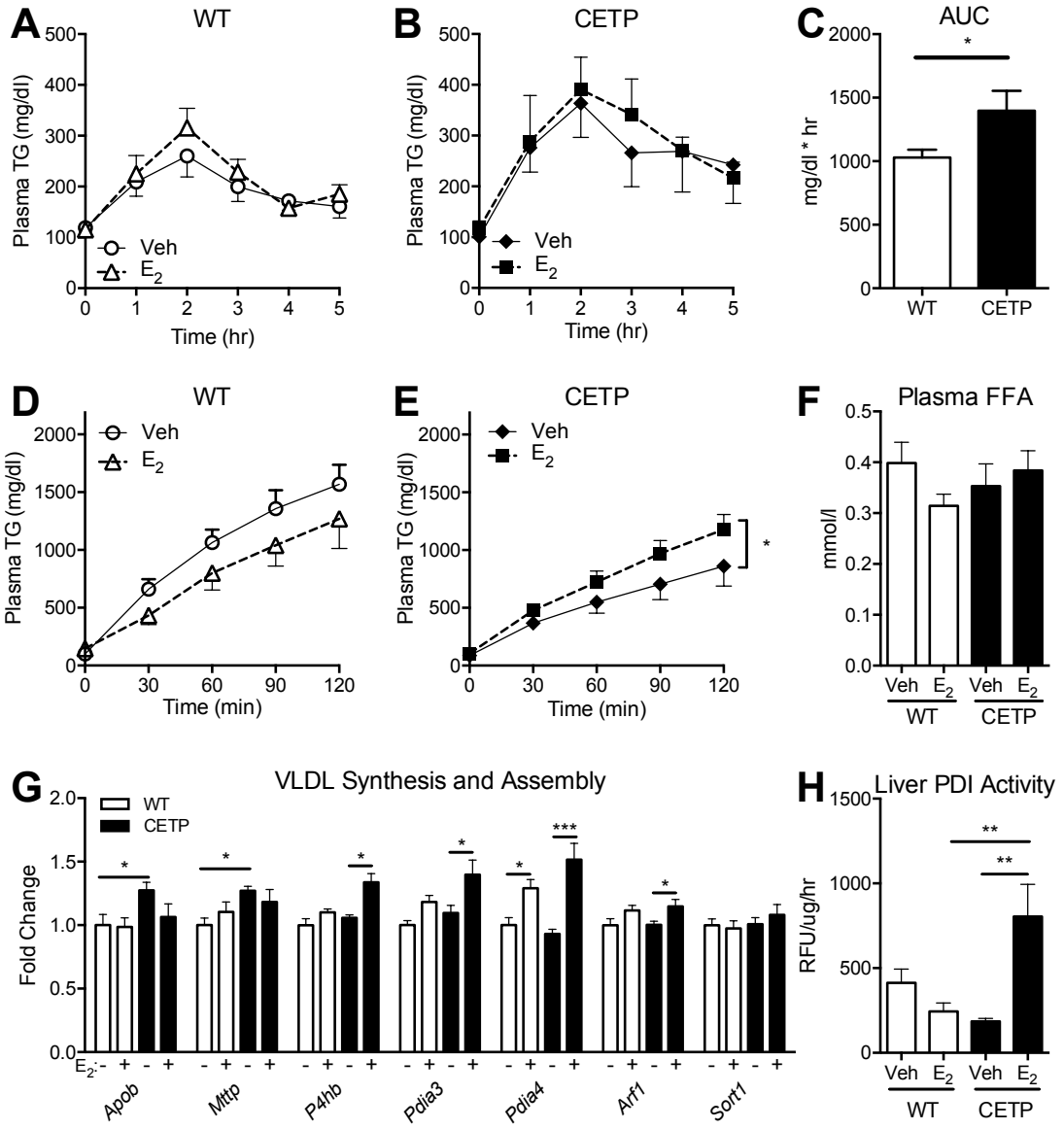

Figure 3.3. Estrogen treatment raises TG production in CETP mice via enhanced expression and activity of genes involved in VLDL synthesis and assembly.
A-B. Plasma TGs following oral TG bolus (200 µl/mouse) in 12hr fasted WT (A) and CETP (B) females treated with Veh or E_2. (Repeated Measures ANOVA, n=5-9/group). **C.** Area under the curve (AUC) analysis of plasma TGs over time after oral fat bolus. *P<0.05 (t-test, n=11-18/group) **D-E.** Plasma TG production after i.v. injection of lipoprotein lipase inhibitor Triton WR-1339 (500 mg/kg) in 3hr fasted WT (D) and CETP (E) females treated with Veh or E_2. *P<0.05 (Repeated Measures ANOVA, n=5-7/group) **F.** Plasma free fatty acid levels (ANOVA, n=7-9/group) in WT or CETP females treated with Veh or E_2. **G.** Liver mRNA expression of VLDL synthesis and assembly targets of mice treated with vehicle (-) or estrogen (+). Liver mRNA expression was determined using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). All genes were normalized to Cyclophilin A and Veh treated WT females were set to 1. *P<0.05, ***P<0.001 (ANOVA, n=7-9/group). **H.** Liver enzymatic activity of Protein Disulfide Isomerase (PDI). **P<0.01 (ANOVA, n=7-9/group). All data are summarized using mean ± SEM.

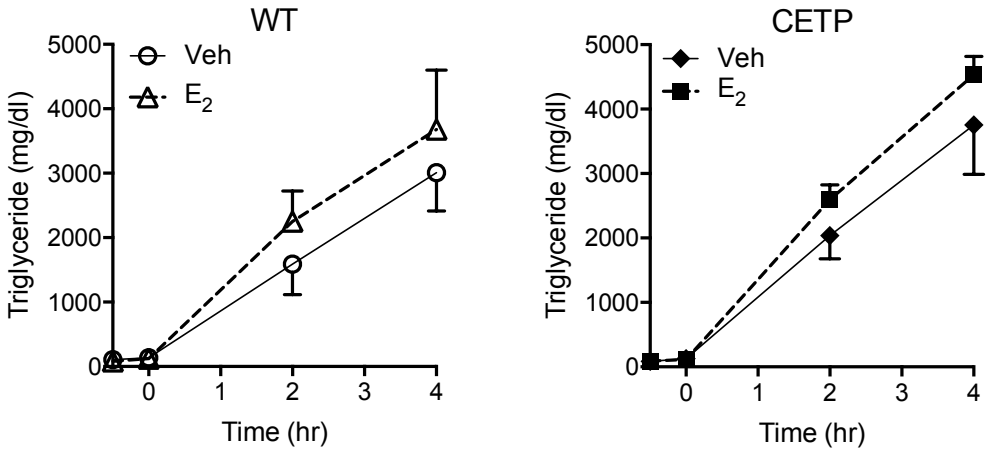

Figure 3.4. Chylomicron TG production did not change with estrogen treatment or with CETP expression.
WT and CETP mice were treated with Veh or E2 24hr prior to study. Following a 12hr fast, mice received an intraperitoneal injection of Triton WR-1339 (500 mg/kg) 30 min prior to olive oil gavage (200 μl/mouse). Plasma TGs were measured over 4hfs from tail blood sampling. Neither CETP nor estrogen significantly altered chylomicron TG production. (Repeated-measures ANOVA, n=4-5/group). All data are summarized using mean ± SEM.

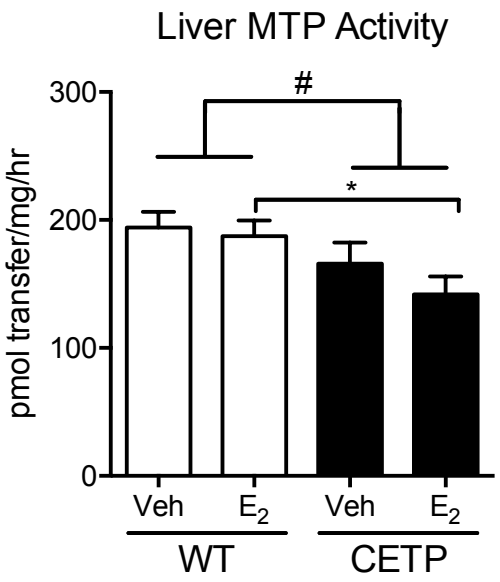

Figure 3.5. Liver MTP activity is lower in CETP mice.
Liver MTP activity was quantified from liver homogenates from Veh or E_2 treated WT and CETP mice. Mice expressing CETP had lower activity of MTP in liver homogenates. $^{\#}P<0.05$ for Genotype effect $^{*}P<0.05$ for post-hoc comparison (2-way ANOVA, n=7-9/group). All data are summarized using mean ± SEM.

CETP lowers liver TG content through enhanced β-oxidation.

Since liver TG is the source for VLDL-TG, we sought to understand if CETP altered liver TG content in these female mice after OVX. Estrogen treatment reduced liver TG content by 70% in WT females (5.82 ± 0.81 *vs.* 1.64 ± 0.56 μg/mg liver, Figure 3.6A). Surprisingly, expression of CETP reduced liver TG content by nearly 60% relative to WT mice (2.46 ± 0.77 *vs* 5.82 ± 0.81 μg/mg liver, CETP Veh *vs*. WT Veh, Figure 3.6A). Estrogen treatment did not further reduce liver TG content in CETP females (Figure 3.6A). Liver cholesterol content did not change with estrogen treatment in either WT or CETP females (Figure 3.6B). Thus, expression of CETP substantially reduced liver TG content. Because liver TG content is a major determinant of VLDL production, this reduced liver TG content likely explains why TG production rates were lower in CETP females compared to WT females.

To determine how CETP reduced liver TG content, we examined markers of β-oxidation, TG synthesis and TG uptake. During prolonged fasting, the liver produces ketone bodies through β-oxidation of fatty acids. Therefore, plasma ketone bodies serve as an *in-vivo* index of liver β-oxidation. After an 18hr fast, CETP females had over twice the levels plasma β-hydroxybutyrate, the most abundant plasma ketone, compared to WT females (Figure 3.6C). Following 5hr of refeeding, plasma β-hydroxybutyrate levels decreased to similar levels in both WT and CETP females (Figure 3.6C). In vehicle treated mice, CETP expression raised mRNA levels of several genes involved in β-oxidation in liver (*Ppara, Cpt2, Acox1, Acadm*, Figure 3.6D), which cumulatively increased β-oxidation *in vivo* as indicated by increased plasma β-hydroxybutyrate levels (Figure 3.6C). Estrogen treatment reduced expression of several β-oxidation targets similarly in WT and CETP mice (Figure 3.6D). Expression of CETP did not substantially reduce expression of genes involved in TG synthesis (Figure 3.7A) or TG uptake and storage (Figure 3.7B), suggesting that these pathways are unlikely to contribute to the reduction in liver TG seen in CETP females. Surprisingly, CETP expression not only blunted the estrogen response of

certain TG metabolic genes (i.e. *Fasn*, Figure 3.7A, *Cd36*, Figure 3.7B), but also promoted new responses to estrogen in other TG metabolic targets (i.e. *Ppara*, Fig 3D, *Srebf2*, Figure 3.7B) that are not seen in WT females. CETP expression did not alter tissue delivery of estrogen to muscle, white adipose or liver (Figure 3.8). Thus, CETP expression caused a differential response to estrogen treatment in several pathways involved in liver TG metabolism without affecting delivery of estrogen to tissues. Furthermore, CETP expression increased liver β-oxidation, which likely explains how CETP expression reduces liver TG content.

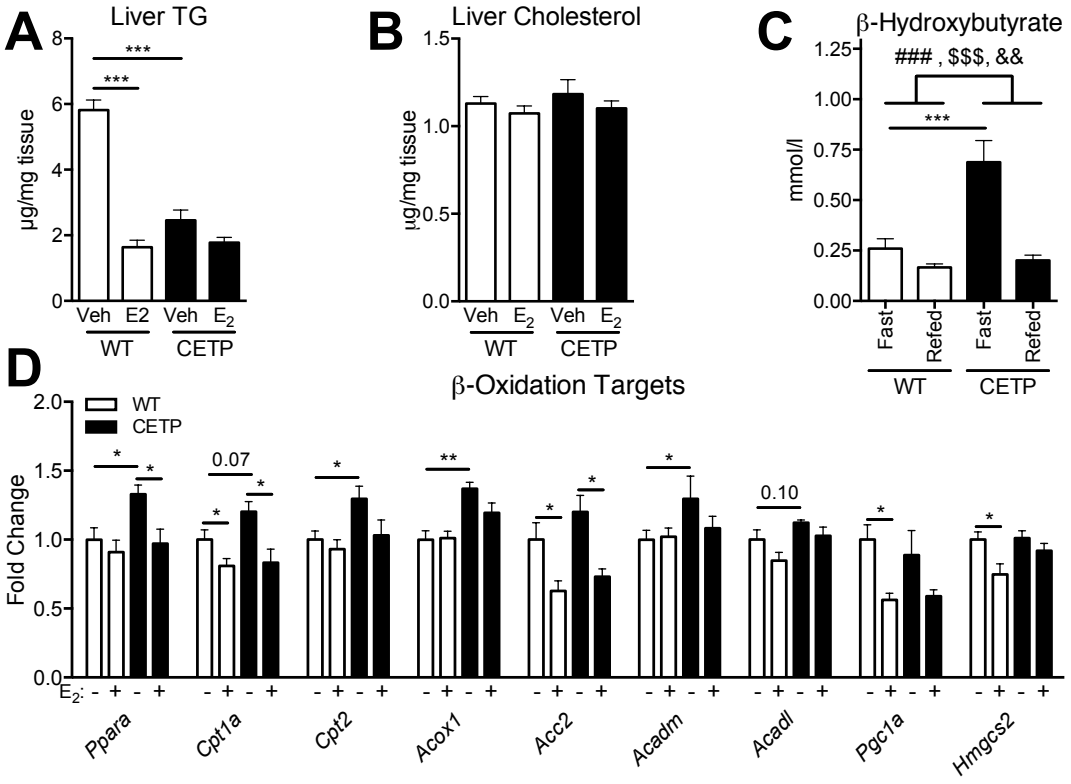

Figure 3.6. Transgenic expression of CETP lowers liver TG content.
A-B. Liver TG (A) and cholesterol (B) content in WT and CETP females treated with Veh or E_2. ***P<0.001 (ANOVA, n=5-7/group). **C.** Plasma β-hydroxybutyrate in 18hr fasted and 5hr refed WT and CETP mice. ***P<0.001, $^{###}$P<0.001 for Genotype effect, $^{\$\$\$}$P<0.001 for Refeeding effect, $^{\&\&}$P<0.01 for Interaction (2-way ANOVA, n=6-9/group). **D.** Liver mRNA expression of β-oxidation targets. *P<0.05, **P<0.01 (ANOVA, n=7-9/group). All data are summarized using mean ± SEM.

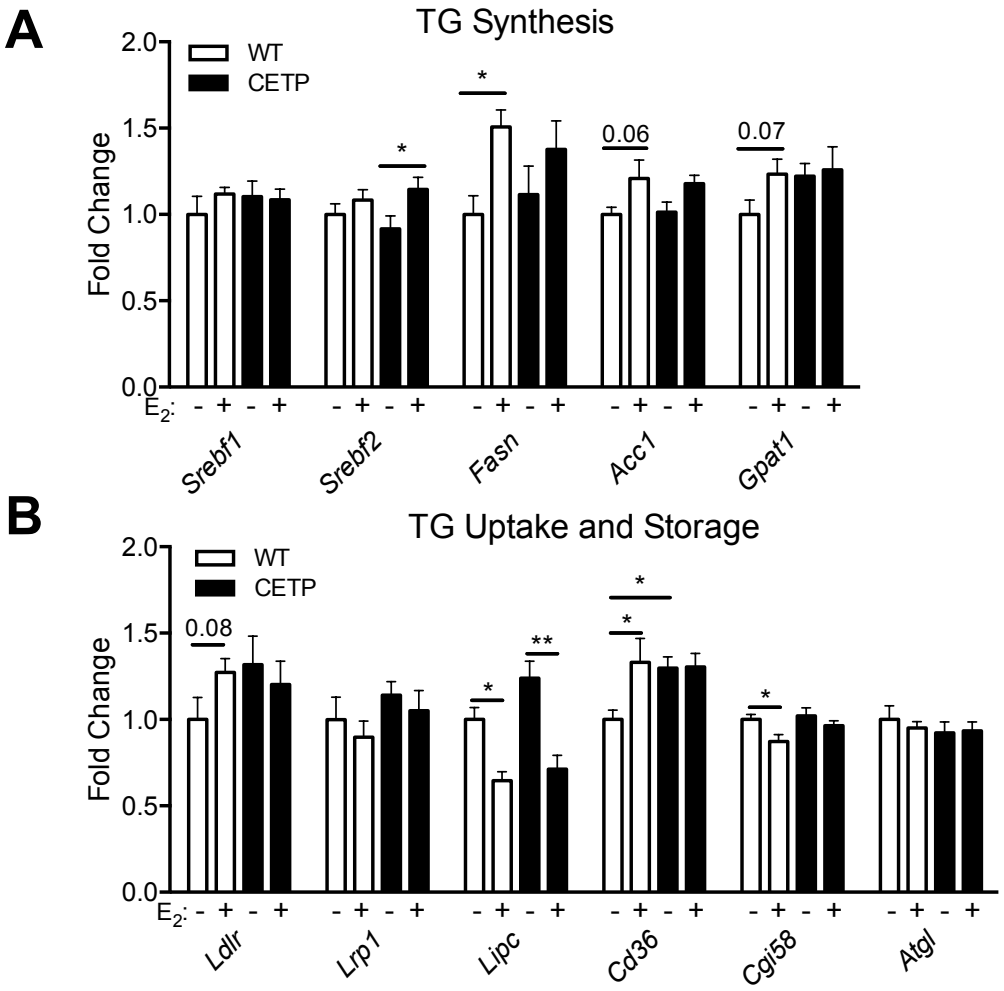

Figure 3.7. CETP expression does not reduce liver mRNA expression of TG synthesis or TG uptake and storage targets.
Liver mRNA expression was measured in WT and CETP females treated with vehicle (-) or estrogen (+) using qRT-PCR. **A.** Liver mRNA expression of TG synthesis targets. *P<0.05 (ANOVA, n=7-9/group). **B.** Liver mRNA expression of TG uptake and storage targets. *P<0.05 **P<0.01 (ANOVA, n=7-9/group). All data are summarized using mean ± SEM.

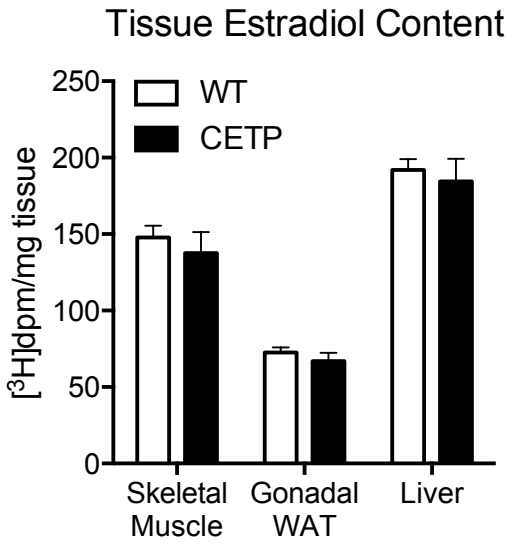

Figure 3.8. CETP does not alter tissue estrogen delivery.
WT and CETP mice were given radiolabeled estrogen ([2,4,6,7-3H(N)]-Estradiol, 20 μCi/mouse, PerkinElmer) along with 1 μg/g estrogen. Tissues were isolated after 24hr and snap frozen in liquid nitrogen. Tissues were dissolved in Solvable (30-200 mg tissue in 2ml at 37ºC overnight, PerkinElmer) and then quantified by scintillation counting. CETP expression did not alter estradiol delivery to skeletal muscle (vastus), parametrial white adipose tissue (gonadal WAT) or liver. All data are summarized using mean ± SEM.

Discussion

It has become increasingly evident that women have a unique set of factors that contribute to risk of cardiovascular disease (273, 274). Understanding the unique aspects of cardiovascular disease risk in women may lead to discovery of novel therapeutics for women, as cardiovascular disease prevention has improved only modestly in women over the last 30 years when compared to men (2). Estrogen has several beneficial effects on risk of cardiovascular disease risk, including reduced plasma glucose levels, reduced plasma insulin levels, and reduced risk of type 2 diabetes. Despite these improvements, estrogen treatment actually raises VLDL-TG production (93-95), which may negate improvements in other cardiovascular disease risk factors. The therapeutic use of estrogen has been limited because of the mixed effects of estrogen has on risk of cardiovascular disease risk. Furthermore, the mechanisms by which estrogen mediates these effects remain unknown.

Here, we provide the first evidence that CETP may underlie the effects estrogen on both glucose and and TG metabolism. Expanding on our previous work demonstrating that CETP expression improved insulin sensitivity in females, we demonstrate that this female specific effect of CETP on glucose metabolism required estrogen. Additionally, we show that CETP is also required for the hypertriglyceridemic effect of estrogen. Specifically, we show that estrogen increases mRNA expression and activity of several genes involved in VLDL synthesis and assembly, especially PDI. Thus, expression of CETP is required for estrogen to raise plasma TGs, increase TG production and increase expression and activity of genes involved in VLDL synthesis and assembly.

In our efforts to understand how CETP contributes to increasing VLDL-TG production with estrogen treatment, we also found that CETP had additional effects on TG metabolism that did not require estrogen treatment. We found that CETP expression reduced liver steatosis by 60% (Figure 3.6A). This reduction in liver steatosis corresponded with increases in liver mRNA

expression of genes involved in β-oxidation and with increases in plasma ketone levels. We also found that CETP expression impaired TG clearance relative to WT littermates (Figure 3.3A-C). Thus, in addition to CETP expression modulating estrogen action on glucose and TG metabolism, CETP can also impact TG metabolism independent of estrogen, which suggests that CETP has a broader role in TG metabolism than previously known.

Previous efforts to understand this pathway may have overlooked the important role of CETP in regulating TG metabolism in females because mice naturally lack CETP. The mouse models used in these studies had genetic presence or absence of transgenic CETP, whereas all humans have CETP. In humans, CETP activity varies 6-8 fold (207), likely due to effects of obesity (275, 276), insulin (277) and estrogen (278) on the regulation of CETP expression. Our transgenic CETP model allowed us to discover that CETP expression facilitates a hypertriglyceridemic response to estrogen, without the confounding effects of estrogen regulation on CETP expression. All females in this study were ovariectomized to remove the contribution of endogenous ovarian hormones and to reduce variability from natural estrus cycling. We examined the effects of estrogen 24hr after treatment to avoid long term changes associated with estrogen treatment, such as reduced adiposity, reduced insulin, and increased plasma free fatty acids, all of which impact TG metabolism (279). Furthermore, although many studies use male mice, our study focused on the effect of estrogen on TG metabolism in females. In agreement with the present study, another group also found that CETP delayed clearance of plasma TGs in female mice (280). Another study found that CETP did not alter TG production or clearance in male ApoE*3-Leiden mice (281). Our results suggest that certain effects of CETP may be dependent on expression in females or treatment with estrogen. We previously showed that CETP can protect against insulin resistance in females, but not in males (254). Further understanding of how CETP alters TG metabolism in males will help identify general effects of CETP expression versus sex-specific or estrogen-specific functions of CETP.

The role of CETP in atherosclerotic cardiovascular disease remains unclear despite several decades of work in this area. CETP activity or mass has correlated both positively (205) and negatively (207, 209) with cardiovascular disease. Genetic polymorphisms in *CETP* have been associated with cardiovascular disease in targeted approaches (212) but have not been associated with cardiovascular disease in genome-wide association studies (132, 217, 219). Mouse models show that CETP improves (233) or worsens (228) measures of atherosclerosis. Whether CETP inhibition reduces cardiovascular disease risk remains to be determined. Although two clinical trials of CETP inhibitors did not reduce cardiovascular disease outcomes (240, 241), and a third was recently halted due to inefficacy, CETP inhibition may ultimately remain a viable therapeutic strategy because of its LDL lowering properties. Our data demonstrate that CETP inhibits clearance of TG and apoB containing chylomicrons. Inhibition of CETP should therefore increase clearance of TG and apoB particles and lower plasma TGs. Recently, a CETP inhibitor was shown to increase clearance of apoB-containing lipoproteins and lowered plasma TGs in humans (282). The failure of several CETP inhibitors may also suggest that CETP has additional functions beyond regulating HDL cholesterol levels.

In conclusion, our work demonstrates that CETP is required for estrogen to increase VLDL-TG production and that CETP has a broader function in TG metabolism in a transgenic mouse model expressing CETP. While CETP-mediated reductions in liver TG content should lower risk of atherosclerosis (283), CETP-mediated impairment of postprandial TG clearance and increases in VLDL production in response to estrogen might increase risk of atherosclerosis (265, 268). Thus, CETP may have both beneficial and harmful effects on cardiovascular disease risk. Furthermore, the dual effect of CETP may explain why CETP inhibitors have not been effective in reducing cardiovascular disease risk. Additional understanding how CETP alters TG metabolism may foster development of more effective therapies to treat cardiovascular disease in humans.

CHAPTER IV

4. CHOLESTERYL ESTER TRANSFER PROTEIN ALTERS LIVER AND PLASMA TRIGLYCERIDE METABOLISM THROUGH TWO DISTINCT LIVER NETWORKS IN FEMALE MICE.

(Adapted from Palmisano *et al. J Lipid Res*. 2016 (264))

Abstract

Women are protected from cardiovascular disease relative to men. Estrogen action contributes to improving a number of risk factors for cardiovascular disease and may contribute to the reduced risk of cardiovascular disease seen in women. Estrogen Receptor α (ERα) is an important mediator of the beneficial effects of estrogen, as demonstrated in *in-vivo* mouse models. The molecular mechanisms of the hypertriglyceridemic effect of estrogen is not well understood, potentially because *in-vivo* mouse models do not recapitulate this effect of estrogen. Previously, we demonstrated that expression of Cholesteryl Ester Transfer Protein (CETP) was required for estrogen to raise VLDL-TG production in females. Additionally, we demonstrated that CETP reduced liver TG content in females through enhanced β-oxidation. Here, we explore the molecular mechanisms required for CETP expression to alter TG metabolism in females. We show that liver ERα was required for CETP to enhance β-oxidation and reduce liver TG content, but was dispensable for estrogen to raise VLDL production. SHP, an important effector of ERα, has been shown to be involved in VLDL production. Liver SHP was required for CETP to increase VLDL production in response to estrogen. Thus, CETP alters at least two networks governing TG metabolism – one involving SHP to increase VLDL-TG production in response to estrogen, and another involving ERα to enhance β-oxidation and lower liver TG content.

Introduction

Women are protected from cardiovascular disease relative to men. At any given age, women have about half the incidence of cardiovascular disease compared to men. Additionally, women have about a ten-year delay in onset of first myocardial infarction compared to men. In women, risk of cardiovascular disease begins to increase at the time of menopause. Furthermore, women with surgical removal of ovaries prior to menopause have increased risk of cardiovascular disease relative to women of the same age. Thus, estrogen is thought to mediate the protective effects against cardiovascular disease.

Estrogen therapy in postmenopausal women improves certain risk factors for cardiovascular disease, but the overall effect on cardiovascular disease is neutral. Estrogen therapy in postmenopausal women contributes to reduced plasma glucose and insulin levels, increased insulin sensitivity and reduced incidence of type 2 diabetes mellitus. Furthermore, estrogen therapy also improves a number of lipid risk factors for cardiovascular disease including reducing total cholesterol, increasing HDL cholesterol, reducing LDL cholesterol, and reducing lipoprotein(a) concentration. Despite these improvements, estrogen treatment also increases plasma TG levels by increasing VLDL-TG production, which may increase risk of cardiovascular disease. This estrogen-mediated increase in plasma TGs may negate improvements in other risk factors. Overall, estrogen treatment has a neutral or harmful effect on cardiovascular disease outcomes.

Estrogen exerts its effects on metabolism via several signaling mechanisms. Canonical estrogen function involves the diffusion of estrogen into cells where they bind Estrogen Receptors α or β (ERα or ERβ) in the cytoplasm. This ligand/receptor binding initiates a conformational change that facilitates receptor translocation into the nucleus, homodimerization and binding to Estrogen-Response Elements (ERE) located in the genome. Following DNA

binding, estrogen receptors alter transcription of target genes by recruitment of coactivators that promote transcription or by recruitment of corepressors the inhibit transcription. Estrogen receptors can also alter transcription of genes lacking EREs through recruitment by other transcription factors to other genomic locations. In addition to the classical effects of estrogens on gene transcription, estrogen can alter cell signaling through receptors localized to the cell surface. ERα has an isoform that localizes to the cell surface to mediate effects on TG metabolism. Additionally, a G-protein coupled estrogen receptor, Gper1 (aka Gpr30), has recently been identified. Lastly, estrogens may exert their metabolic effects by altering expression of other transcription factors, like SHP, that regulate gene expression independent of estrogen receptor action.

Estrogen has been shown to exert its beneficial metabolic effects primarily through ERα (106, 108, 112, 284). In mouse models, estrogen has been shown to reduce food intake and reduce adiposity through the action of ERα (284). The ability of estrogen to improve insulin sensitivity is dependent on liver ERα function (106, 109). Furthermore, estrogen has been shown to reduce liver steatosis through the action of liver ERα (109, 285, 286). The role of liver ERα in regulating the hypertriglyceridemic effect of estrogen is currently unknown, partly because mouse models do not readily recapitulate this effect. Previously, we demonstrated that expression of CETP is required for the hypertriglyceridemic effect of estrogen, but the mechanisms for this are unknown. Here, we explore the molecular mechanisms require for CETP to alter TG metabolism. We show that ERα is required for the ability of CETP to enhance β-oxidation reduce liver steatosis. ERα, however, was dispensable for the ability of CETP to raise VLDL in response to estrogen. An important effector of estrogen action, Small Heterodimer Partner (SHP), has been shown to be involved in VLDL production. We demonstrate that liver SHP was required for CETP females to increase VLDL in response to estrogen. Thus, CETP alters at least two networks governing TG metabolism – one involving

ERα to enhance β-oxidation and lower liver TG content, and another involving SHP to increase VLDL-TG production in response to estrogen.

Results

Liver Estrogen Receptor α is required for CETP to reduce liver TG content.

We demonstrated that CETP expression in mice causes several novel effects on both liver TG metabolism and plasma VLDL-TG production in response to estrogen, but the molecular mechanisms responsible for this effect are unknown. ERα is the predominant estrogen receptor expressed in the liver (287) and regulates a number of lipid metabolic pathways in the liver (110, 286). To test the hypothesis that liver ERα is required for CETP expression to alter TG metabolism, we bred CETP transgenic mice onto a congenic strain with a hepatocyte specific deletion of ERα (LKO-ERα) (109). Whereas CETP expression decreased liver TG nearly 60% relative to WT controls (Figure 3.6A), deletion of liver ERα completely prevented CETP-mediated lowering of liver TG content relative to LKO-ERα controls (Figure 4.1A). Additionally, CETP expression did not increase plasma levels of β-hydroxybutyrate in the absence of liver ERα (Figure 4.1B). Furthermore, in the absence of liver ERα, CETP failed to increase β-oxidation gene expression (*Ppara, Cpt2, Acox1, Acadm*) with vehicle or estrogen treatment (Figure 4.1C). Thus, liver ERα is required for CETP expression to lower liver TG content and increase β-oxidation.

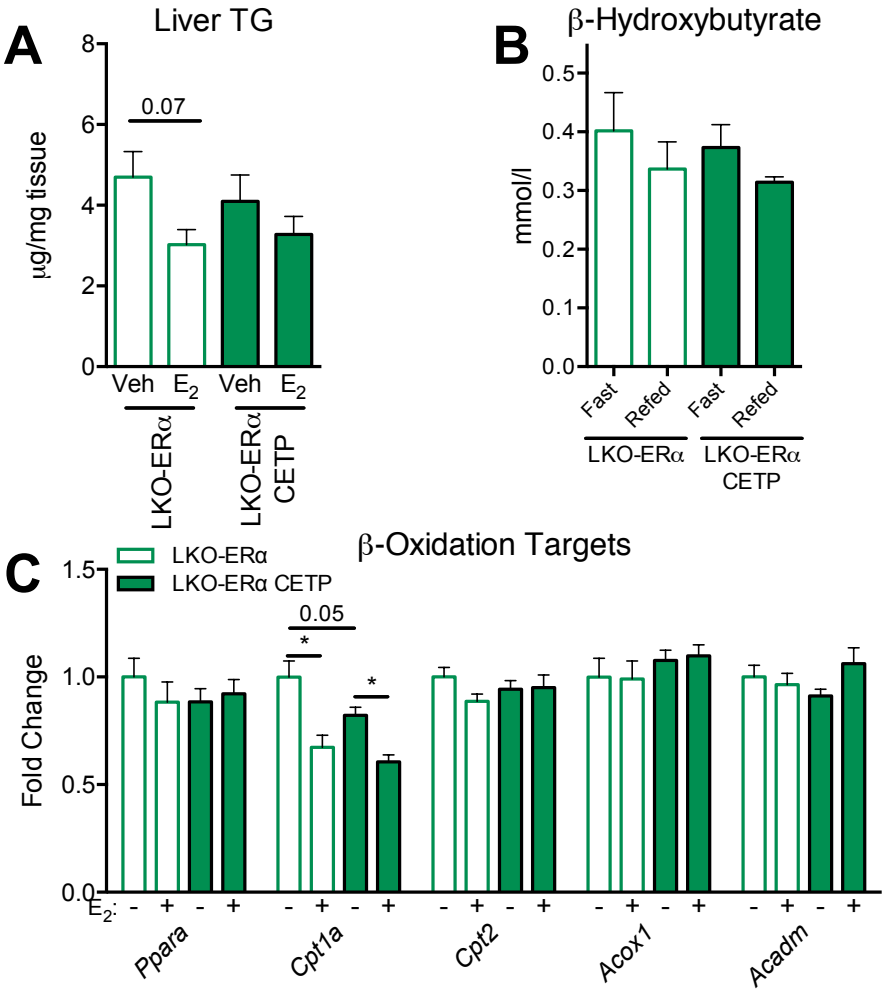

Figure 4.1. Liver ERα is required for CETP to lower liver TG content.
A. Liver TG content in mice with liver-specific knockout of Estrogen Receptor α (LKO-ERα) or mice with liver-specific knockout of Estrogen Receptor α with CETP (LKO-ERα CETP) females treated with Veh or E_2. (ANOVA, n=7-8/group) **B.** Plasma β-hydroxybutyrate in 18hr fasted and 5hr refed LKO-ERα and LKO-ERα CETP females (n=6-8/group). **C.** Liver mRNA expression of β-oxidation targets. *P<0.05 (ANOVA, n=7-8/group). All data are summarized using mean ± SEM.

Liver Estrogen Receptor α is not required for CETP to raise TG production in response to estrogen.

We next determined if CETP expression also required liver ERα to increase plasma TGs and TG production in response to estrogen. Estrogen treatment did not raise plasma cholesterol or lipoprotein cholesterol content in LKO-ERα or LKO-ERα CETP mice (Figure 4.3A-C). CETP expression resulted in the expected reduction in HDL cholesterol content in LKO-ERα CETP females (Figure 4.2C). Despite deletion of liver ERα, estrogen treatment raised plasma TGs in LKO-ERα CETP females, whereas estrogen treatment did not alter plasma TGs in LKO-ERα females (Figure 4.3D). In addition, estrogen treatment dramatically raised VLDL-TG in LKO-ERα CETP females, but estrogen only modestly increased VLDL-TG in LKO-ERα CETP females (Figure 4.2E-F). Also, estrogen raised liver PDI activity in LKO-ERα CETP but not LKO-ERα females (Figure 4.3A), which indicates that estrogen may be able to raise VLDL production even in the absence of liver ERα. Estrogen did not alter TG production in LKO-ERα females (Figure 4.3B). However, estrogen treatment raised TG production in LKO-ERα CETP females (Figure 4.3C). Thus, liver ERα is not required for CETP to raise TG production in response to estrogen.

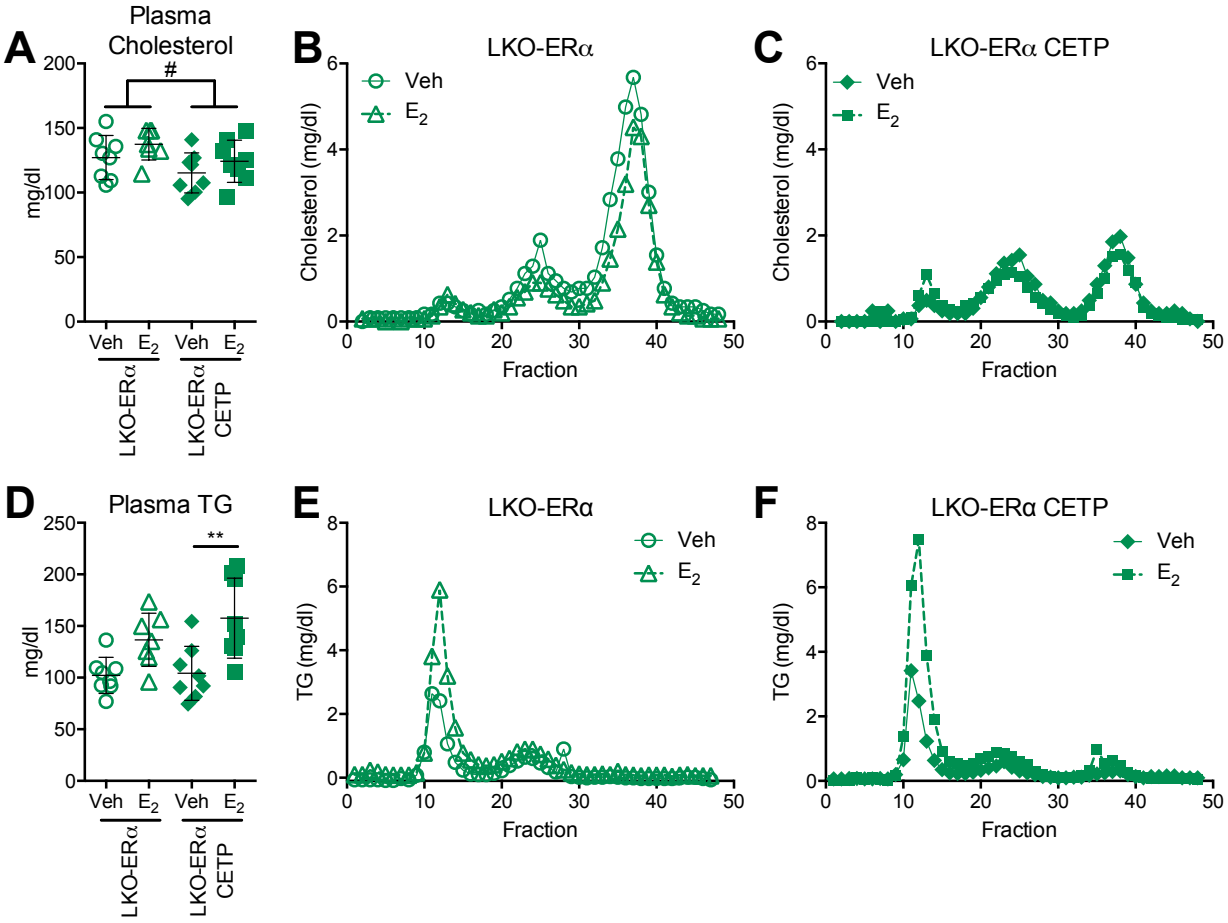

Figure 4.2. Liver ERα is not required for CETP expression to raise plasma TG and VLDL-TG in response to estrogen.

A. Plasma Cholesterol in LKO-ERα and LKO-ERαCETP females after Veh or E_2 treatment. ($^{\#}P<0.05$ for Genotype effect, 2-way ANOVA, n=7-8/group). **B-C.** Cholesterol content of FPLC separation of plasma lipoproteins in LKO-ERα (B) and LKO-ERαCETP (C) females after Veh or E_2 treatment (pooled plasma from n=7-8/group). **D.** Plasma TG in LKO-ERα and LKO-ERαCETP females after Veh or E_2 treatment (**P<0.01, ANOVA, n=7-8/group). **E-F.** Cholesterol content of FPLC separation of plasma lipoproteins in LKO-ERα (E) and LKO-ERαCETP (F) females after Veh or E_2 treatment (pooled plasma from n=7-8/group). All data are summarized using mean ± SEM.

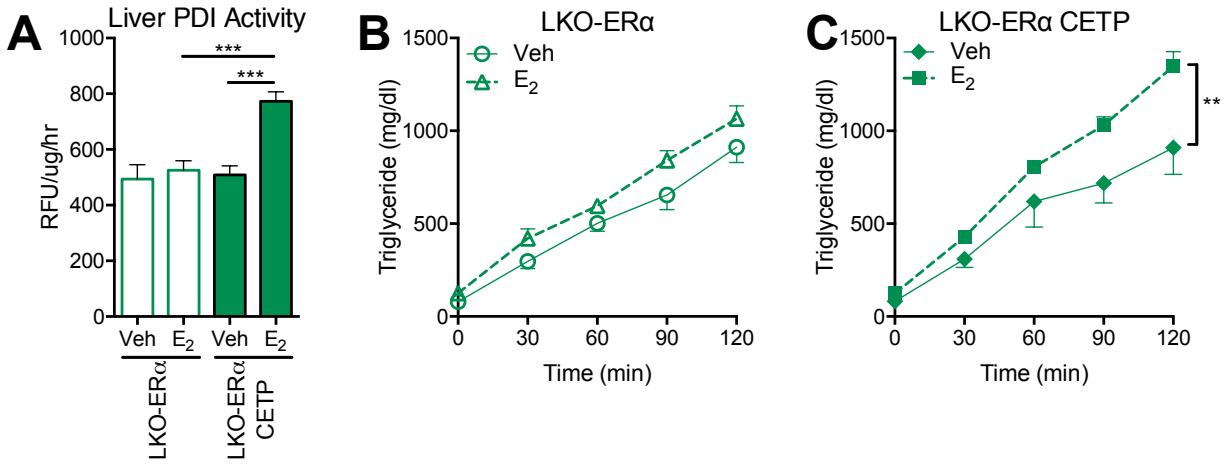

Figure 4.3. Liver ERα is not Required for CETP to Raise VLDL Production with Estrogen. **A.** Liver PDI activity in LKO-ERα and LKO-ERα CETP females treated with Veh or E_2 (***P<0.001, ANOVA, n=7-8/group). **B-C.** TG production after i.v. injection of Triton WR-1339 (500 mg/kg) in 3hr fasted LKO-ERα (B) and LKO-ERα CETP (C) females treated with Veh or E_2 (**P<0.01, Repeated Measures ANOVA, n=4-7/group). All data are summarized using mean ± SEM.

These data indicate that estrogen may raise VLDL production in mice expressing CETP via another estrogen receptor in liver, like the G-Protein Coupled Estrogen Receptor, Gper1 (also known as Gpr30). To test the hypothesis that estrogen signals via Gper1 to raise VLDL production in CETP expressing mice, LKO-ERα and LKO-ERα CETP mice were pre-treated with a Gper1 antagonist prior to treatment with estrogen. Gper1 antagonism prevented estrogen from raising TG production in LKO-ERα CETP mice (Figure 4.4), indicating that estrogen may signal through Gper1 to raise VLDL production in mice expressing CETP. Taken together, these data demonstrate that liver ERα is dispensable for estrogen-mediated increases in plasma TGs and TG production in CETP mice, but that liver ERα is required for CETP-mediated increases in β-oxidation and concomitant lowering of liver TG content.

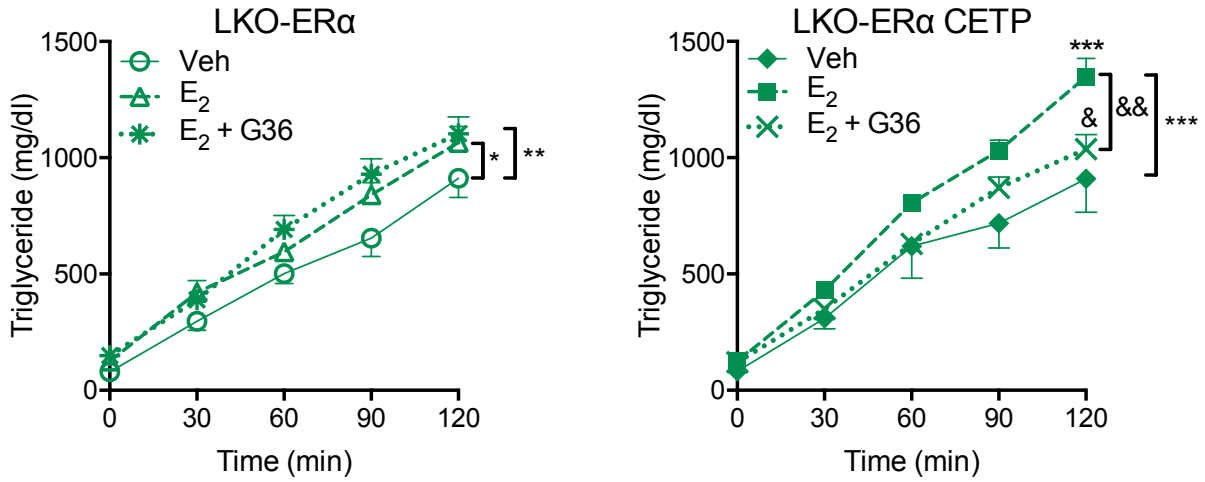

Figure 4.4. Gper1 Antagonism Prevents Estrogen-Mediated Increases in TG Production in LKO-ERα CETP Mice.

LKO-ERα and LKO-ERα CETP mice were given veh, E_2 or pretreated with a Gper1 antagonist (G-36, 5 μg/g, Cayman Chemical) for 1 hr and then given E_2. Following 24hr of treatment, mice fasted 3 hr and then given i.v. Triton WR-1339 (500 mg/kg). Plasma TG concentration was measured over 2 hrs. Pretreatment with a Gper1 antagonist significantly reduced estrogen-mediated increases in TG production in LKO-ERα CETP mice. $^{\&}P<0.05$ $^{\&\&}P<0.01$ (vs E_2), *P<0.05 **P<0.01 ***P<0.001 (vs Veh), Repeated-Measures ANOVA (n=4-8/group). All data are summarized using mean ± SEM.

Liver Small Heterodimer Partner is required for CETP to increase TG production with estrogen treatment.

Since liver ERα was not required to raise TG production in response to estrogen treatment in CETP mice, we sought to determine additional nuclear factors required for this effect in CETP mice. Previously, we showed that CETP expression enhanced bile acid signaling to the nuclear receptor SHP in females (254). SHP regulates a number of metabolic pathways, including VLDL-TG production (288) and estrogen signaling (289, 290). Estrogen is also known to increase liver SHP expression in mice (290). We found that estrogen increased SHP mRNA in the liver of CETP mice (Figure 4.5A). Estrogen also increased liver SHP mRNA in WT females, but this was not statistically significant. We also found that SHP regulates liver mRNA expression of several PDI isoforms (*P4hb, Pdia3*, Figure 4.5B). Since estrogen induces expression of both SHP and PDI in CETP mice, and since we found that SHP regulates liver PDI expression, we hypothesized that SHP may be required in CETP females to induce PDI and increase TG production in response to estrogen.

To test the hypothesis that CETP requires liver SHP to raise plasma TG production in response to estrogen treatment, we bred CETP transgenic mice onto a congenic strain with a hepatocyte specific deletion of SHP (LKO-SHP, Figure 4.5B). Estrogen did not alter plasma cholesterol levels or lipoprotein cholesterol content in either LKO-SHP or LKO-SHP CETP females (Figure 4.6A-C). CETP expression resulted in the expected reduction in HDL cholesterol content in LKO-SHP CETP females (Figure 4.6C). In the absence of liver SHP, estrogen treatment failed to raise plasma TGs or increase VLDL-TG in females with CETP (Figure 4.6D-F). Additionally, estrogen treatment also failed to raise TG production in females expressing CETP in the absence of liver SHP (Figure 4.6E-F). In fact, TG production decreased with estrogen treatment in both LKO-SHP and LKO-SHP CETP mice. This decrease in TG

production may be due to estrogen-mediated increases in liver bile acid levels (291), which is known to reduce liver TG production (292). In addition to the effects on plasma TG metabolism, liver SHP deletion prevented estrogen-mediated increases in liver PDI activity (Figure 4.7C, compared to Figure 3.3H). Finally, deletion of liver SHP prevented estrogen-mediated increases in mRNA expression of genes of VLDL synthesis and assembly in CETP females (Figure 4.7D, compared to Figure 3.3G). Thus, liver SHP expression is required for estrogen to increase plasma TGs, TG production, and mRNA expression and activity of genes involved in VLDL assembly in CETP females.

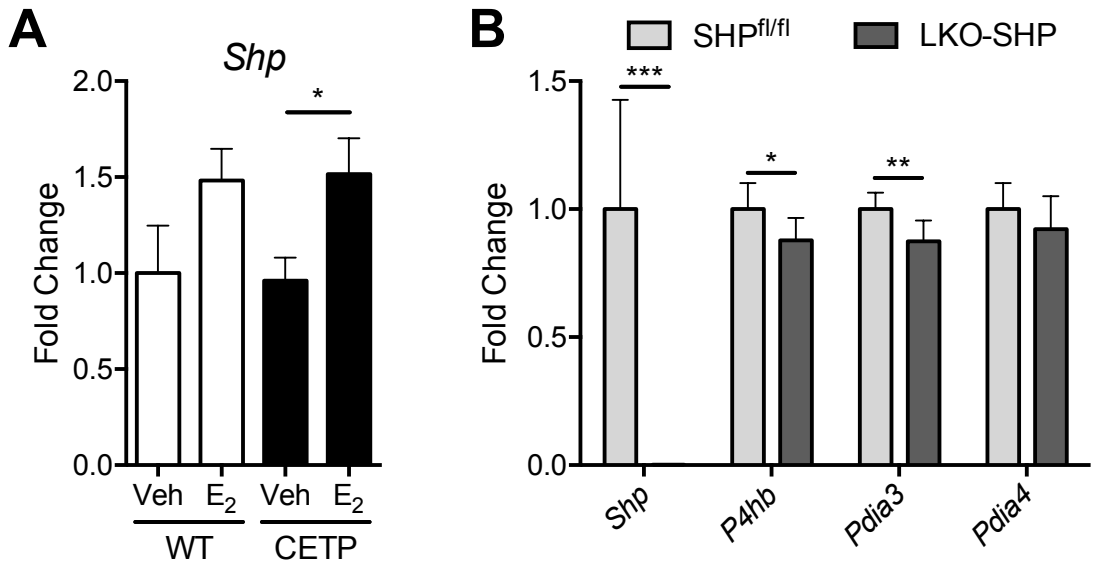

Figure 4.5. CETP enhances estrogen-induced expression of SHP, a regulator of PDI. **A.** CETP enhances estrogen-mediated induction of liver Small Heterodimer Partner (*Shp*) mRNA expression. WT or CETP female mice were given veh or E_2 and mRNA expression of *Shp* was measured using qRT-PCR. *P<0.05 (ANOVA, n=7-9/group) **B.** Liver mRNA expression of isoforms of PDI were measured in mice with liver-specific knockout of SHP (LKO-SHP). Liver SHP regulates mRNA expression PDI isoforms in liver. *P<0.05, **P<0.01, ***P<0.001 (ANOVA, n=7-9/group). All data are summarized using mean ± SEM.

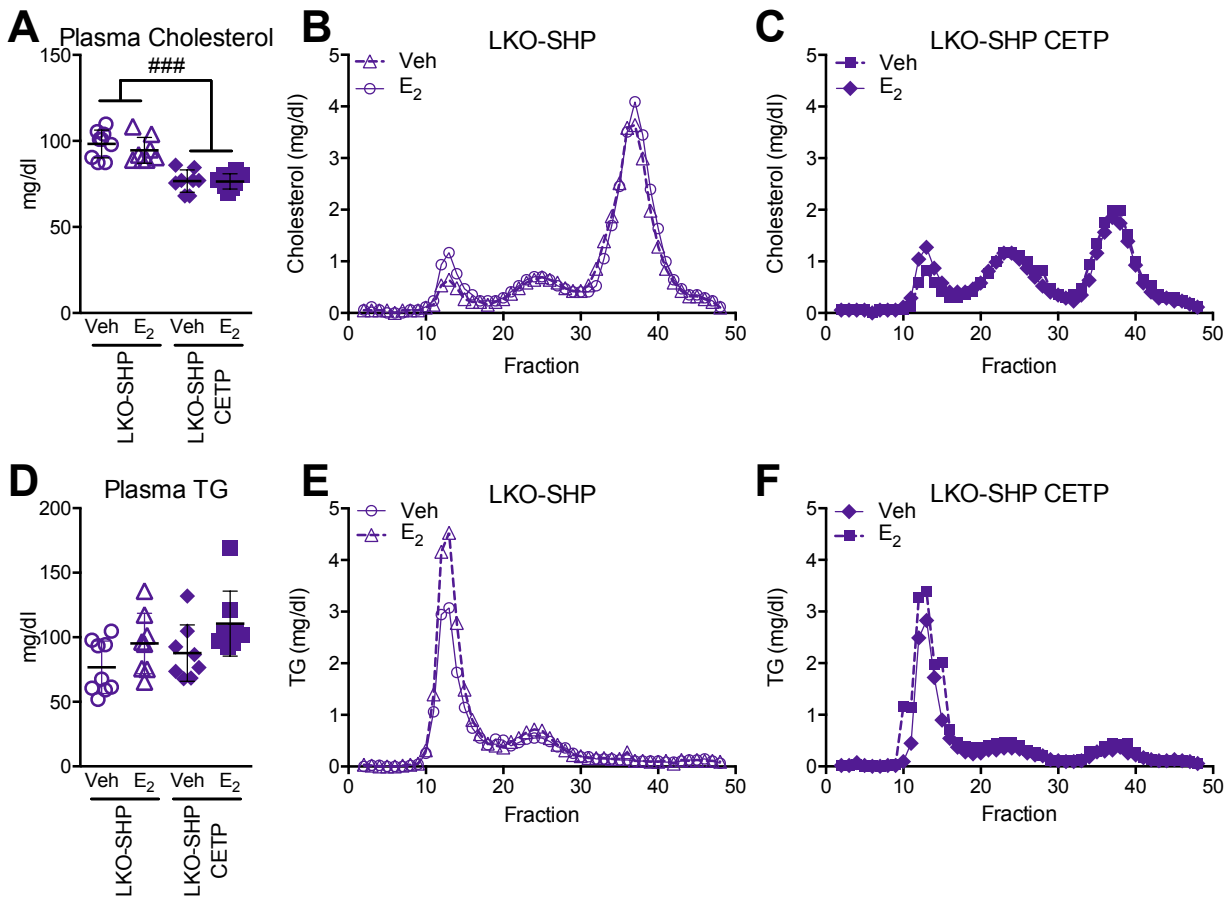

Figure 4.6. Liver SHP is required for CETP to raise plasma TG and VLDL-TG with estrogen treatment.

A. Plasma Cholesterol in LKO-SHP and LKO-SHP CETP females after Veh or E_2 treatment. ($^{###}P<0.001$ for Genotype effect, 2-way ANOVA, n=8/group). **B-C.** Cholesterol content of FPLC separation of plasma lipoproteins in LKO-SHP (B) and LKO-SHP CETP (C) females after Veh or E_2 treatment (pooled plasma from n=7-8/group). **D.** Plasma TG in in LKO-SHP and LKO-SHP CETP females after Veh or E_2 treatment (n=8/group). **E-F.** TG concentration of FPLC separation of plasma lipoproteins in LKO-SHP and (E) and LKO-SHP CETP (F) females after Veh or E_2 treatment (pooled plasma from n=8/group). All data are summarized using mean ± SEM.

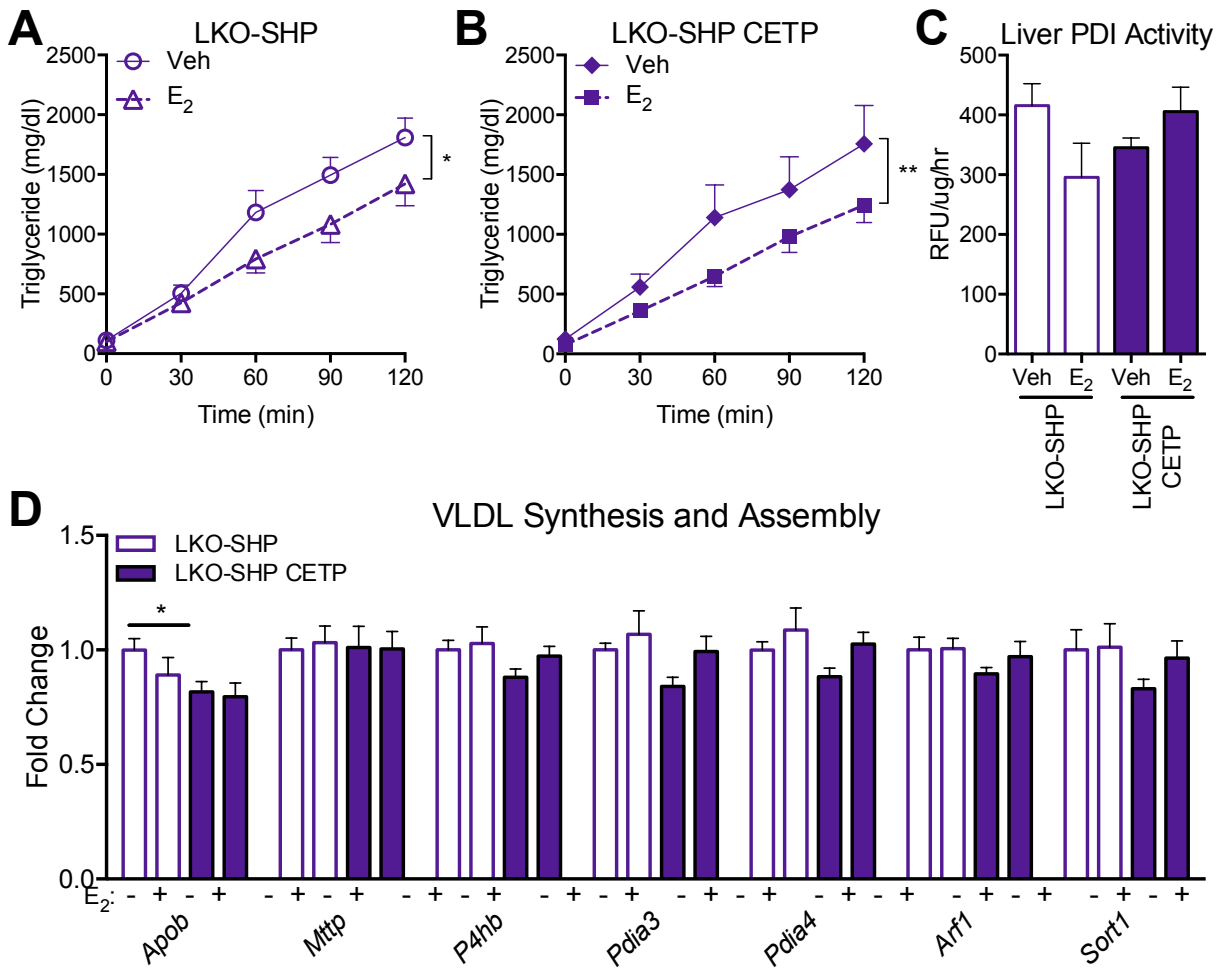

Figure 4.7. Liver SHP is required for CETP to increase VLDL production in response to estrogen in females.

A-B. TG production after i.v. injection of Triton WR-1339 (500 mg/kg) in 3hr fasted LKO-SHP (A) and LKO-SHP CETP (F) females treated with Veh or E_2. (*P<0.05 **P<0.01, Repeated Measures ANOVA, n=6/group). **C.** Liver PDI activity in LKO-SHP or LKO-SHP CETP females after Veh or E_2 treatment (n=8-9/group). **D.** Liver mRNA expression of VLDL synthesis and assembly targets (*P<0.05, ANOVA, n=8/group). All data are summarized using mean ± SEM.

We next tested if liver SHP was required for CETP expression to alter liver TG content. Similar to a previous report (289), deletion of liver SHP prevented estrogen-mediated reductions in liver TG content in females (Figure 4.8A). Whereas CETP expression previously lowered liver TG content by 60% relative to WT mice (Figure 3.6A), expression of CETP in the absence of liver SHP actually raised liver TG content by 35% relative to LKO-SHP females (4.70 ± 0.74 *vs* 3.46 ± 0.50 μg/mg, LKO-SHP CETP veh *vs*. LKO-SHP veh, $P<0.05$, Figure 4.8A). Because liver fat content is a determinate of VLDL production, this increase in liver TG content likely contributed to raising TG production in LKO-SHP CETP to similar levels as LKO-SHP mice, while CETP previously reduced TG production relative to WT mice (Figure 3.3D-E). This increased liver TG content was also associated with reduced mRNA levels of *Acox1*, a gene involved in liver β-oxidation (Figure 4.8B), but not mRNA levels of other β-oxidation genes. Despite higher liver TG content, liver mRNA expression of lipoprotein uptake receptors *Ldlr* and *Lrp1* were reduced in LKO-SHP CETP relative to LKO-SHP females (Figure 4.8C). Additionally, estrogen treatment lowered liver TG content in LKO-SHP CETP females (Figure 4.8A). Estrogen treatment did not alter liver cholesterol content in either LKO-SHP or LKO-SHP CETP females (Figure 4.8D). Thus in the absence of liver SHP, CETP expression did not reduce liver TG content. In fact, CETP expression raised liver TG content in the absence of liver SHP, which was subsequently reduced in response to estrogen. Taken together, these data indicate that liver SHP is required for CETP to raise plasma TGs and TG production in response to estrogen treatment.

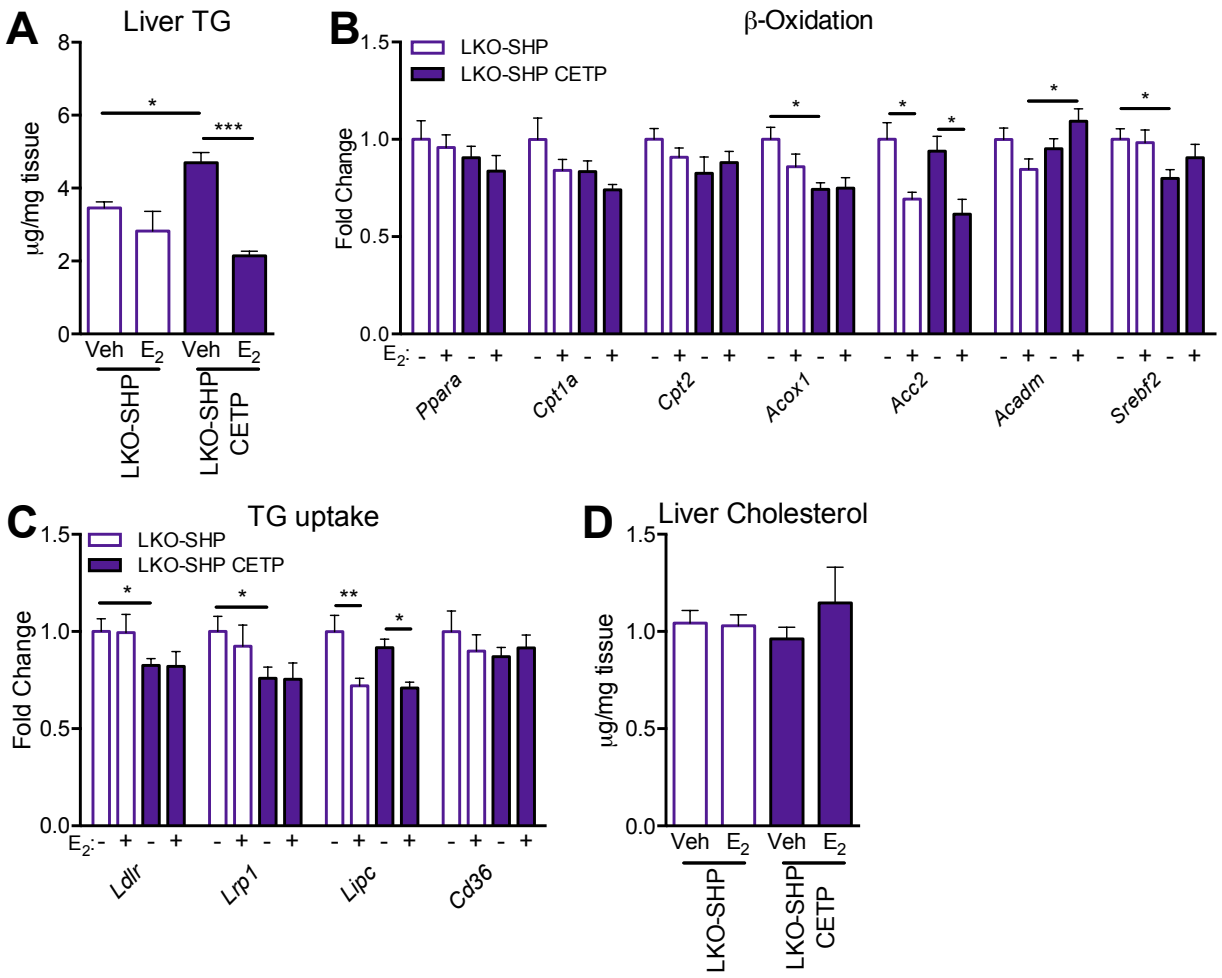

Figure 4.8. Liver SHP is required for CETP to lower liver TG content.
A. Liver TG content in female mice with liver-specific deletion of Small Heterodimer Partner (LKO-SHP) and LKO-SHP mice expressing transgenic CETP (LKO-SHP CETP) given vehicle (Veh) or estrogen (E_2). CETP fails to lower liver TG content in the absence of liver SHP. *P<0.05, ***P<0.001 (ANOVA, n=7-9/group). **B-C.** Liver mRNA expression of β-oxidation targets (B) and TG uptake targets (C) in LKO-SHP and LKO-SHP CETP females given vehicle (-) or estrogen (+). *P<0.05 **P<0.01 (ANOVA, n=8/group) **D.** Liver cholesterol content of LKO-SHP and LKO-SHP CETP females given vehicle (Veh) or estrogen (E_2). (n=7-9/group). All data are summarized using mean ± SEM.

Discussion

In an effort to understand the molecular mechanisms by which CETP alters TG metabolism, we found that CETP alters two distinct liver signaling networks. We found that liver ERα was required for CETP to enhance β-oxidation and subsequently lower liver steatosis. Surprisingly, liver ERα was not required for estrogen to raise TG production in CETP mice, suggesting that estrogen may signal through another less-highly expressed estrogen receptor to increase TG production. We found that Gper1 signaling and liver SHP expression were required for estrogen to raise TG production in CETP females. Additionally, liver SHP was not required for CETP to lower liver steatosis. In fact, in the absence of liver SHP, CETP raised liver TG content, opposite of what CETP does with normal liver SHP signaling. These results suggest that CETP disrupts at least two distinct nuclear signaling networks to alter TG metabolism.

Since CETP alters TG metabolism in females, we hypothesized that liver ERα would be a major upstream regulator of the ability of CETP to alter TG metabolism. ERα is a major regulator of lipid metabolism in the liver and is the predominant estrogen receptor expressed in the liver (110, 286, 287). We found that ERα was required for CETP to lower liver steatosis, but not to raise liver TG production in response to estrogen. This was surprising for two reasons. Firstly, the ability of CETP to lower liver steatosis occurred in vehicle treated CETP females, which is when estrogen levels were low. This suggests that CETP may alter ERα function even in the absence of estrogen ligand interaction with ERα. Secondly, ERα was not required for CETP to raise VLDL production in response to estrogen. This suggests that CETP enhances estrogen action through a less highly expressed estrogen receptor or a novel signaling pathway to raise VLDL production.

In our efforts to understand how CETP expression increased VLDL production in response to estrogen, we found that Gper1 and liver SHP played an important role in this pathway. We found that inhibition of Gper1 signaling prevented CETP from raising VLDL production in

response to estrogen. This suggests that Gper1 signaling in response to estrogen contributes to increasing VLDL production and raising TG levels in CETP mice. Gper1 knockout mice have increased plasma TGs, suggesting that Gper1 signaling reduces plasma TGs (123). Thus, CETP expression has a novel, inverse effect on Gper1 signaling compared to normal mice. In addition to Gper1, we found that liver SHP was required for CETP to raise VLDL production in response to estrogen. SHP is a nuclear receptor involved in many metabolic pathways, including glucose and lipid metabolism. SHP has been shown to be an important regulator of VLDL production. Deletion of SHP in mice increases VLDL production, suggesting that SHP signaling acts to reduce VLDL production. Our data suggest that liver SHP is required to increase VLDL production in response to estrogen in CETP mice, indicating that SHP exerts opposite effects on VLDL production depending on the presence of CETP and estrogen. Since both Gper1 and liver SHP were required to raise VLDL in response to estrogen in CETP females, Gper1 might contribute to regulation of SHP function, especially in CETP mice. A previous report showed that Gper1 agonists can regulate liver SHP(293), but further work will be needed to confirm this relationship in CETP mice. Thus, CETP requires both Gper1 signaling and liver SHP to increase VLDL production in response to estrogen, but CETP expression seems to reverse the known function of these receptors.

How CETP modulates nuclear receptor function remains an unanswered question. Especially perplexing is that CETP is a secreted protein and nuclear receptors ERα and SHP are intracellular. Further complicating the picture is that CETP seems to reverse the normal function of Gper1 and SHP. CETP may alter intracellular signaling by a number of mechanisms. One way CETP may alter liver signaling networks is by facilitating delivery of lipid signaling molecules to receptors on intracellular organelles. Indeed, CETP can facilitate lipid transfer between organelles *in-vitro* (294). Another way CETP may alter liver signaling is by augmenting pathways dependent on LDL and VLDL. Transgenic expression of CETP in mice, which naturally lack CETP, increases plasma LDL and VLDL levels (Figure 3.1D-E). This may

augment signaling pathways dependent on apoB-containing lipoproteins. A final way CETP may alter liver signaling may be through indirect effects in other tissues. CETP expression may alter adipose function in response to estrogen and alter secretion of adipokines that indirectly regulate VLDL metabolism (295-297). Overexpression of CETP has been shown to alter adipocyte TG content (298, 299). Each of these potential methods by which CETP may alter signaling within the liver may ultimately alter the function of ERα or SHP directly through posttranslational modifications or indirectly by altering the available transcription factor milieu or the function of other transcription factors. The net effect of the changes CETP has on cellular signaling ultimately results in differential responses to estrogen. Thus, CETP likely functions upstream of ERα and SHP to alter cell signaling, which ultimately leads to changes affecting the ability of these receptors to regulate target gene expression. Although we do not yet fully understand all the molecular events connecting CETP to ERα or SHP, this work provides an important step forward in understanding the specific functions and signaling pathways required by CETP to alter TG metabolism.

Since we show that CETP has estrogen-specific effects on TG metabolism, CETP may underlie certain estrogen-specific responses to TG metabolism in humans. Hormone replacement therapy raises plasma TGs in postmenopausal women (84, 300). Moreover, estrogen contributes to elevations in TGs by increasing VLDL production (93-95). A true test of whether CETP facilitates estrogen-mediated increases in TGs in humans would be to compare women with and without CETP deficiency before and after estrogen replacement therapy. Genetic deficiencies in CETP, however, are extremely rare. One study, however, found that genetic polymorphisms in *CETP* modified the effect of hormone therapy on plasma lipoproteins (301). Two additional studies found sex-dependent effects of *CETP* polymorphisms on other aspects of TG metabolism. One found that *CETP* polymorphisms modified the effect of gender on postprandial TG clearance (302). Another study found that *CETP* polymorphisms increased

risk of fatty liver disease in women but not in men (303). Taken together, our data along with human genetic data suggest that CETP may function more broadly in TG metabolism and may underlie certain sex-specific effects, especially in women with estrogen treatment.

In conclusion, this work demonstrates that CETP is signals through two distinct networks to alter TG metabolism – one involving ERα and the enhancement of β-oxidation to reduce liver TG content, and another involving liver SHP and Gper1 to promote VLDL production in response to estrogen. The distinct signaling networks altered by CETP highlights the complex effect of CETP on cell signaling. CETP seems to have a major effect on sex hormone signaling in females, and further work will determine if CETP can alter sex hormone signaling in males. Despite the failure of CETP inhibitors to improve risk of cardiovascular disease in several recent clinical trials, further efforts to develop more selective CETP inhibitors may improve their efficacy. Development of inhibitors that target the negative effects of CETP (i.e. inhibition of TG clearance), but permit the beneficial effects of CETP (i.e. improved glucose tolerance, reduced liver steatosis) may improve the efficacy CETP inhibitors at reducing cardiovascular disease risk. It is currently unknown if certain CETP inhibitors have selective action on these distinct effects of CETP. Further understanding of how CETP alters TG metabolism will facilitate discovery of novel pathways that allow for more specific therapies to treat cardiovascular disease in humans.

CHAPTER V

5. CHOLESTERYL ESTER TRANSFER PROTEIN ALTERS GONADAL HORMONE ACTION TO IMPAIR TRIGLYCERIDE CLEARANCE IN MALE MICE

Abstract

Plasma triglycerides (TGs) are an important risk factor for cardiovascular disease. Both overproduction and impaired clearance of TGs increase plasma TGs and contribute to risk of cardiovascular disease. Understanding mechanisms that regulate TG clearance will yield novel therapeutic targets that may ameliorate risk of cardiovascular disease. Although many secreted proteins have been shown to regulate TG clearance, the effect of lipid transfer proteins like Cholesteryl Ester Transfer Protein (CETP) on TG clearance remains unknown. We previously demonstrated that expression of CETP in mice, which naturally lack CETP, was required for estrogen to raise VLDL production and increase plasma TGs in females. We also previously demonstrated that CETP expression leads to several estrogen-independent effects on TG metabolism in females. Whether these estrogen-independent effects of CETP on TG metabolism are generalizable to males remains to be determined. Here we explore the role of CETP expression on TG metabolism in male mice. We demonstrate that CETP expression increases plasma TGs, increases TG content of VLDL, and impairs plasma TG clearance in males compared to wild-type (WT) mice lacking CETP. This impaired TG clearance was associated with reduced liver expression of low-density lipoprotein receptor (LDLR) in CETP males. Furthermore, gonadal hormones were required for CETP to impair TG clearance and to reduce liver LDLR expression in CETP males. Thus, expression of CETP in males impairs TG

clearance and raises plasma TGs in males via a mechanism involving gonadal hormone regulation of liver LDLR expression. Further understanding of how CETP influences androgen hormone action to impair TG clearance may lead to discovery of novel targets that may reduce risk of cardiovascular disease.

Introduction

Elevated plasma triglycerides (TGs) are an important risk factor for cardiovascular disease (146, 149, 150, 152, 153). Both overproduction of TG and reduced clearance of TGs contribute to increasing TG levels and increasing risk of cardiovascular disease (154, 156, 158, 159, 304). TGs are also an important risk factor for Type 2 Diabetes (160, 161), which is an independent risk factor for cardiovascular disease. The role of plasma triglycerides in risk of cardiovascular disease has been controversial because insulin resistance and obesity, risk factors for both Type 2 Diabetes and cardiovascular disease, also increase TGs. Thus, multifactorial models may underestimate the independent risk of TGs on cardiovascular disease. Human genetic approaches have confirmed that lifelong changes in TGs due to genetic changes contribute to risk of cardiovascular disease (178). Additionally, phenotyping according to plasma TG response to an oral high-fat bolus has clearly demonstrated that postprandial TG clearance predicts risk of atherosclerosis (154-156, 159, 304). Mechanisms regulating TG clearance, therefore, represent a unique therapeutic opportunity to reduce plasma TGs and ameliorate risk of cardiovascular disease.

TG clearance is regulated by a number of secreted proteins and tissue receptors (141). Intestinally absorbed triglycerides are packaged into large apolipoprotein B (apoB) containing lipoproteins called chylomicrons. Once in systemic circulation, chylomicron TGs are hydrolyzed by lipases like lipoprotein lipase (LPL) and hepatic lipase (HL) for uptake of TG into tissues (139, 140). Lipase action and TG removal from these chylomicrons results in conversion to smaller particles called chylomicron remnant particles. Chylomicron remnant particles are cleared by the low-density lipoprotein receptor (LDLR), the LDLR Related Protein 1 (LRP1) and other uptake receptors (135, 137, 138). In addition to clearing chylomicron remnant particles, LDLR and LRP1 regulate clearance of other apoB containing lipoproteins like VLDL and LDL. Impaired expression of these uptake receptors or lipases can decrease TG uptake and

contribute to increased TG levels. Increased or decreased receptor expression will enhance or impair TG uptake. In addition to regulation of TG clearance by uptake receptors, secreted proteins, largely made by the liver, can both stimulate and inhibit TG clearance. Proteins associated with chylomicrons, VLDL, LDL and other lipoproteins act as ligands to receptors expressed by tissues to facilitate lipid uptake by that tissue. Apolipoprotein CII (ApoC2) and Apolipoprotein E (ApoE) facilitate binding of lipoproteins to LPL and LDLR or LRP1, respectively, and promote TG uptake (141). Other secreted proteins like Apolipoprotein CI (ApoC1), Apolipoprotein CIII (ApoC3), Angiopoeitin Like 3 (Angptl3) and Angiopoeitin Like 4 (Angptl4) bind to and inhibit activity of LPL, and impair TG clearance (141). Thus, secreted proteins and tissue expression of lipoprotein receptors contribute to regulation of plasma TG clearance. The role of secreted lipid transfer proteins like Cholesteryl Ester Transfer Protein (CETP) in regulating TG clearance is unknown.

Previous work by in our lab and by other groups suggests that transgenic expression of CETP in mice impacts glucose and TG metabolism with female-specific and estrogen-specific effects (254, 264). Additionally, in humans, certain female-sex diminishes the association between genetic variation in CETP with cardiovascular disease (256-259). In women, changes in CETP is inversely associated with plasma glucose after bariatric surgery (305) and plasma from women with high CETP activity had increased cholesterol efflux capacity relative to women with low CETP activity (306). In men, CETP may have a neutral (307) or positive (308) correlation with measures of glucose and lipid metabolism in. This suggests that CETP may be harmful in males, but specific mechanisms by which CETP increases risk of cardiovascular disease remain unknown.

Here we determine the role of CETP in regulating TG clearance in male mice. Study of CETP function *in-vivo* is hampered by the fact that mouse models naturally lack CETP. Expression of the CETP transgene in mice results in a more human-like lipoprotein distribution (309). Efforts to understand the role of CETP in atherosclerosis have yielded mixed results,

likely due to genetic manipulations required to generate atherosclerosis in mice (225-233). Previous work has shown that CETP improves plasma cholesterol metabolism (310), but studies investigating the role of CETP in triglyceride metabolism are lacking. We previously demonstrated that CETP is required for estrogen to raise VLDL production in females and that CETP reduces liver TG content in females (264). The role of CETP in regulating TG metabolism in males is unknown. Here we demonstrate that expression of CETP in male mice increases plasma TGs by impairing TG clearance. This effect seems to be due to reduced liver lipoprotein receptor expression. We also demonstrate that gonadal hormones are required for CETP to impair TG clearance and for CETP to reduce liver lipoprotein receptor expression in males. Thus, CETP expression in males increases plasma TGs by impairing TG clearance via a mechanism that requires gonadal hormones.

Results

CETP expression increases plasma TG in VLDL in male mice.

We have previously shown that transgenic expression of CETP in female mice has both estrogen-dependent effects and estrogen-independent effects on TG metabolism. To determine if estrogen-independent effects are generalizable to males, we examined the impact of CETP expression on TG metabolism in CETP transgenic male mice and non-transgenic, wild-type (WT) littermates on maintained on chow diet. Expression of CETP did not alter body weight in males (Figure 5.1A). CETP lowered plasma cholesterol levels in CETP males (Figure 5.1B). This CETP-mediated lowering of plasma cholesterol levels is in agreement with its known function on lowering HDL-cholesterol levels and has been previously been shown before in transgenic mice. Expression of CETP increased plasma TGs over 60% in males (157.1 ± 26.9 *vs*. 94.8 ± 17.8 mg/dl, $P<0.001$, Figure 5.1C). As expected from the reduced plasma cholesterol levels, the cholesterol content of HDL fractions was markedly reduced in CETP males as measured by FPLC (Figure 5.1D). CETP expression resulted in a nearly 2-fold enrichment of TG content of VLDL fractions in males as measured by FPLC (Figure 5.1E). CETP expression also modestly increased the TG content of HDL fractions. Thus, transgenic expression of CETP increases plasma TG in VLDL in male mice.

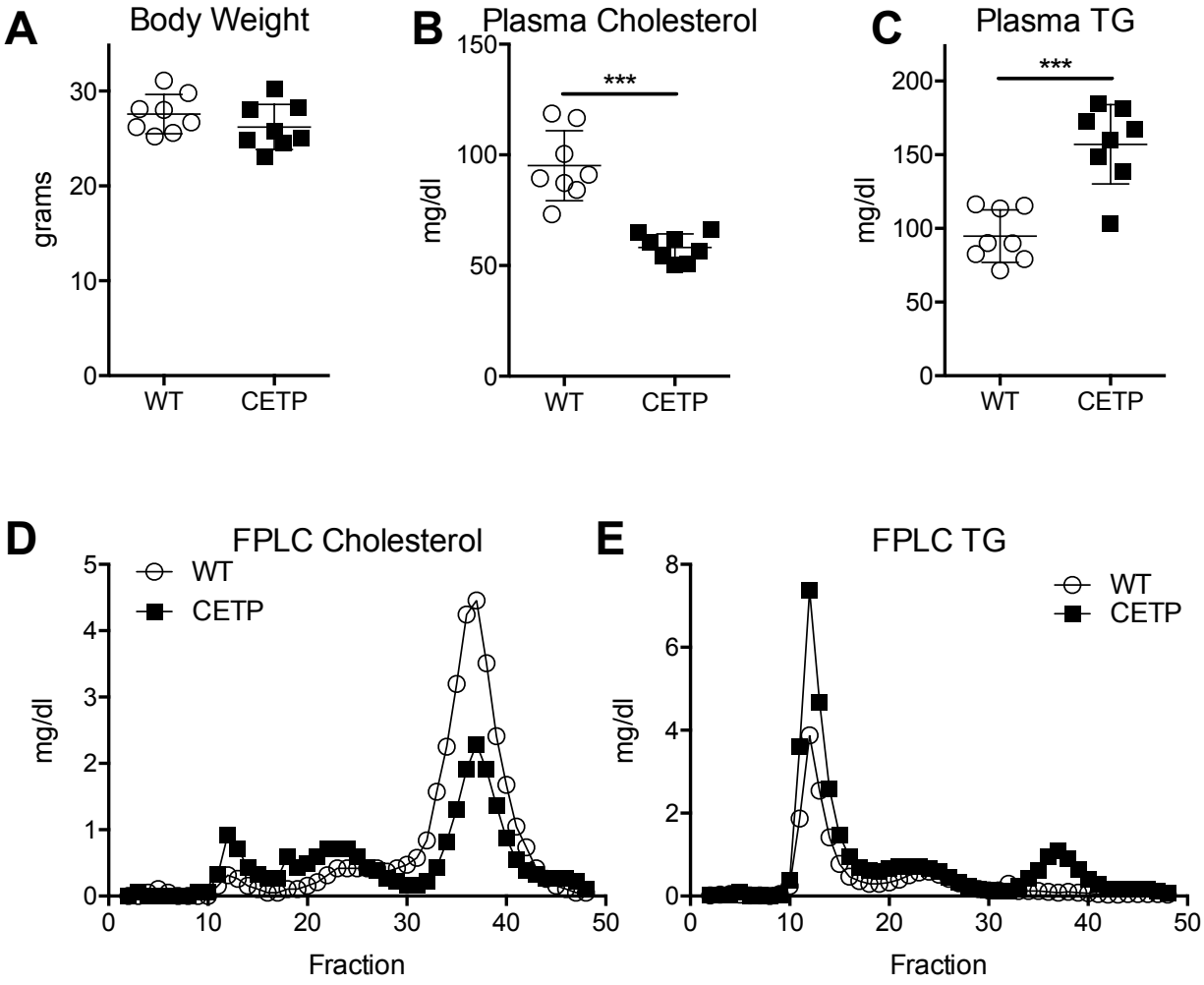

Figure 5.1. CETP Expression Increases Plasma TGs in Males.
A-C. Body weight (A), plasma cholesterol (B), plasma TG (C) of ad libitum fed CETP and WT male mice on chow diet. ***P<0.001 (Student's t-test, n=8/group) **D-E.** Cholesterol content (D) and TG content (E) in each fraction fast protein liquid chromatography (FPLC) separation of pooled plasma lipoproteins (pooled plasma n=8/group). All data are summarized using mean ± SEM.

CETP raises plasma TG by impairing postprandial TG clearance.

To determine how CETP increases plasma TGs and VLDL-TG in males, we measured plasma clearance and production of TGs. TG production was measured in fasted mice after administration of the lipoprotein lipase inhibitor, Triton WR-1339. Expression of CETP did not alter TG production in male mice (Figure 5.2A-B). Clearance of postprandial TGs was measured after an oral olive oil bolus in 12hr fasted mice. CETP expression in males resulted in a greater postprandial TG excursion relative to WT males (Figure 5.2C). Area under the curve (AUC) was more than 80% higher in CETP relative to WT males (Figure 5.2D). This suggests that CETP expression delays clearance of postprandial TGs in males. This is in agreement with the estrogen-independent effect of CETP in on postprandial TG excursion in females. Thus, CETP raises plasma TGs by delaying clearance of postprandial TGs without altering TG production in males.

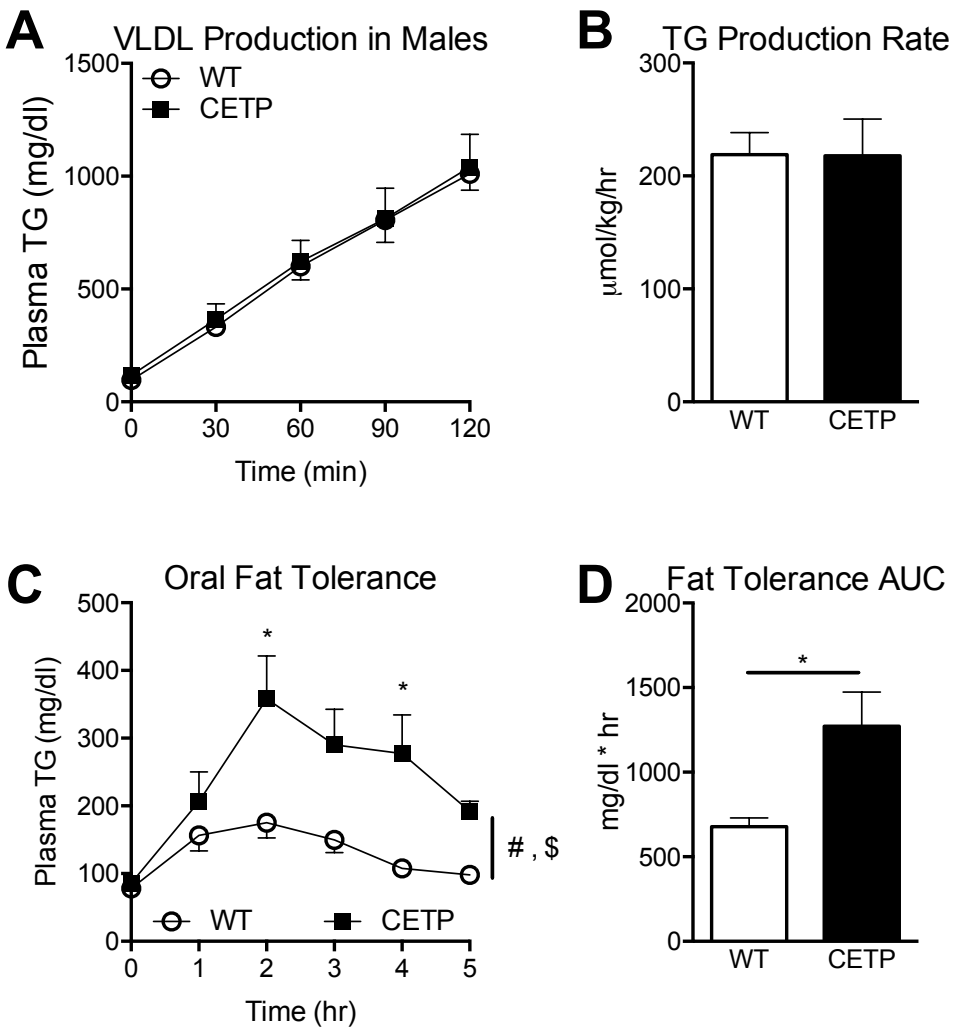

Figure 5.2. CETP raises plasma TGs via delayed clearance of postprandial TGs.
A. Plasma TG production after intravenous injection of lipoprotein lipase inhibitor Triton WR-1339 (500 mg/kg) in 3hr fasted WT and CETP males. **B.** TG production rate **C.** Plasma TGs following oral TG bolus (200 μl/mouse) in 12hr fasted WT and CETP males. $^{\#}P<0.05$ for Genotype effect, $^{\$}P<0.05$ for Interaction effect, $^{*}P<0.05$ for post-hoc comparisons of individual time points (2-way ANOVA). **D.** Area under the curve (AUC) analysis of oral fat tolerance test. All data are summarized using mean ± SEM.

CETP alters plasma apolipoprotein concentrations and liver lipoprotein uptake receptor expression in males.

To understand the molecular mechanisms responsible for the ability of CETP expression to impair postprandial TG clearance in males, we examined the plasma levels and liver expression of proteins governing TG clearance. As expected based on increased VLDL levels, CETP expression resulted in an increased expression of plasma apolipoprotein B (ApoB, Figure 5.3A-B). Additionally, CETP expression reduced plasma levels of apolipoprotein E (Apo E, Figure 5.3A-B). ApoE is an apolipoprotein that facilitates binding to LDLR and LRP1 to mediate uptake of chylomicrons, VLDL and LDL. Reduced expression of ApoE has been shown to impair TG clearance. Here, reduced levels of ApoE likely resembles an underestimate of the effect of CETP since ApoE-containing lipoproteins are less likely to be cleared from circulation in the presence of CETP expression. Expression of CETP did not significantly alter plasma levels of other apolipoproteins (Figure 5.3A-B). Thus, expression of CETP reduces levels of plasma apolipoproteins that govern TG clearance in males.

In addition to the effects of CETP on plasma apolipoprotein levels, we also wanted to understand whether CETP altered expression of receptors governing lipoprotein uptake. CETP expression resulted in reduced liver expression of LDLR and SRB1 (Figure 5.3C-D). LDLR is a major regulator of TG clearance. This reduced expression of LDLR likely explains how TG clearance is impaired and liver cholesterol levels are reduced in CETP males. Surprisingly, CETP reduced liver protein expression of PCSK9 (Figure 5.3C-D). PCSK9 is an important regulator of LDLR protein stability. Increased PCSK9 reduces LDLR levels and reduced PCSK9 increases LDLR levels. Thus, CETP reduces liver LDLR protein independent of PCKS9. Thus, expression of CETP reduces liver expression of proteins important in regulating lipoprotein and plasma lipid uptake. Taken together, these data suggest that the ability of CETP to impair postprandial TG metabolism is a result of reduced levels of plasma apolipoproteins and liver receptors governing TG uptake.

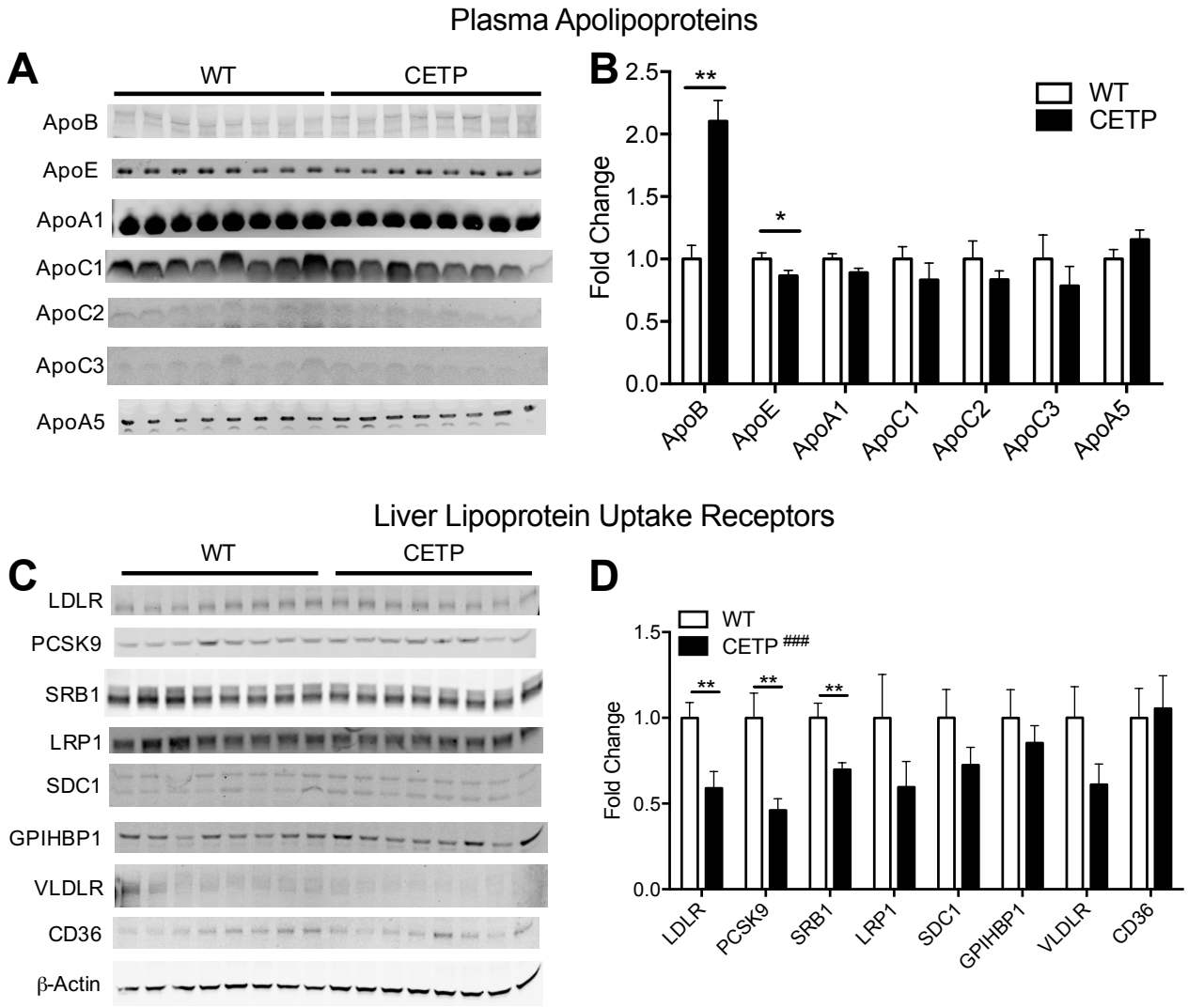

Figure 5.3. CETP reduces plasma apolipoproteins and liver lipoprotein receptors. **A-B.** Western blotting (A) and quantification (B) of plasma apolipoproteins (n=8/group). **C-D.** Western blotting (A) and quantification (B) of liver lipoprotein and lipid uptake receptors (**P<0.01 ANOVA, ###P<0.001 for genotype effect by 2-way ANOVA, n=8/group). All data are summarized using mean ± SEM.

CETP does not alter liver TG content in males.

We previously demonstrated that expression of CETP in female mice reduces liver TG content by 60% relative to WT females. This effect was evident when estrogen levels were low (vehicle treatment), suggesting that the effect of CETP on liver steatosis may be estrogen-independent. To determine if CETP can lower liver TG content independent of male or female sex, we measured liver lipid content in WT and CETP male mice. CETP expression modestly reduced liver cholesterol content by about 10% in males (Figure 5.4A). CETP expression did not alter liver TG content in males relative to WT littermates (Figure 5.4B). In contrast to our previous findings in females (264), expression of CETP did not result in increased fasting levels of plasma β-hydroxybutyrate in males (Figure 5.4C). To understand whether CETP altered cholesterol metabolism, we examined the expression of proteins involved in cholesterol synthesis and catabolism. CETP did alter liver protein expression of Srebp1, Srebp2 or Cyp7a1 (Figure 5.4D-E). The reduced liver cholesterol content, therefore, is likely due to reduced cholesterol uptake as a consequence of reduced LDLR expression (Figure 5.3C-D). Thus, expression of CETP in males does not alter liver lipid content as it does in females. Additionally, expression of CETP in males has a modestly beneficial effect on liver cholesterol content.

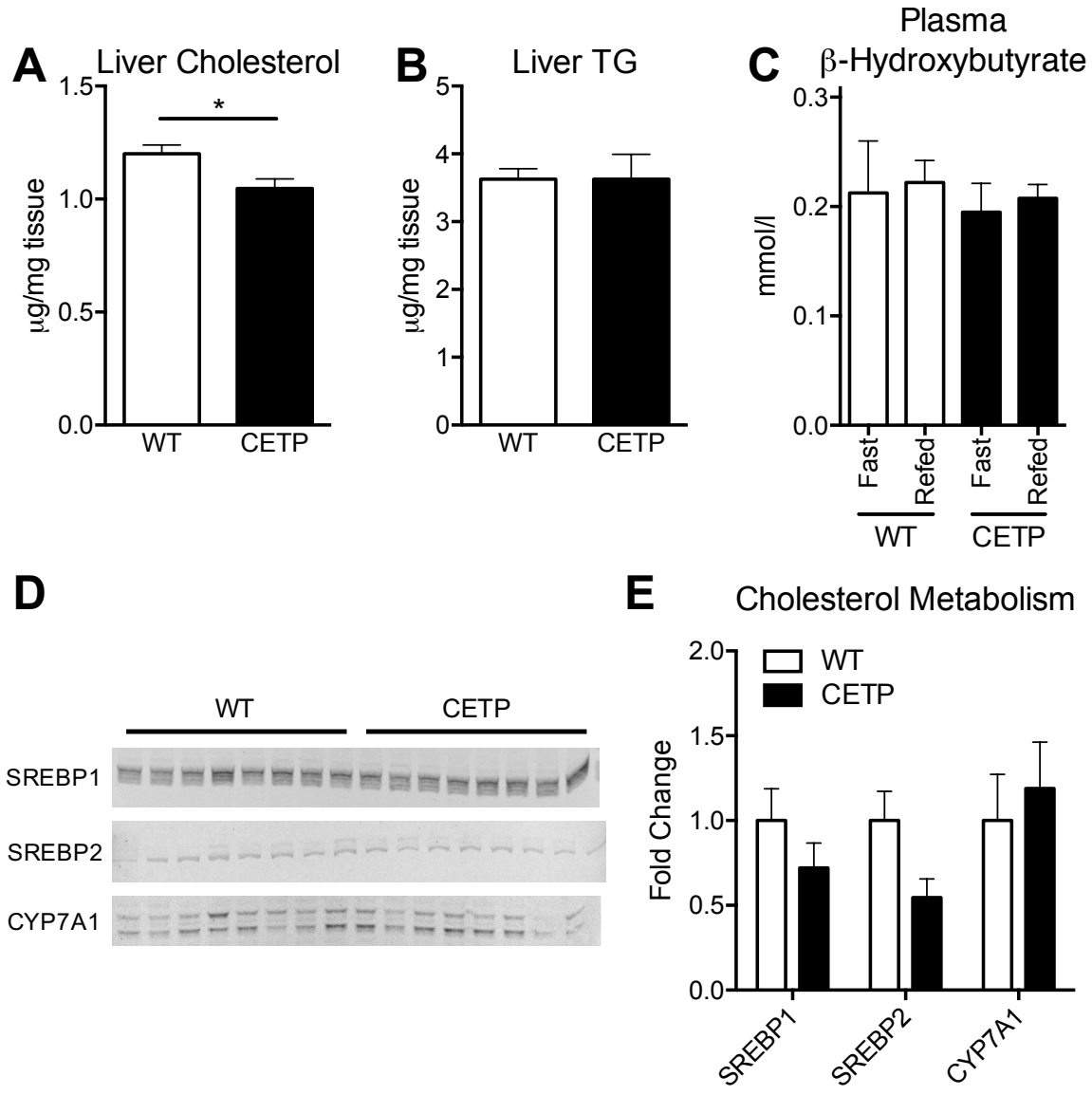

Figure 5.4. CETP does not alter liver TG content in males.
A-B. Liver cholesterol (A) and TG content (B) in WT and CETP males. *P<0.05 (n=8/group) **C.** Plasma levels of β-hydroxybutyrate after 18hr fasting and 5-6hr refeeding (n=7-8/group). **D-E.** Liver western blotting (D) and quantification (E) of proteins regulating liver cholesterol metabolism (n=8/group).

CETP requires gonadal hormones to raise plasma TGs in males.

Sex steroids were required for CETP to raise plasma TGs in females (264). Here, we sought to determine if sex steroids were also required to raise plasma TGs in males following gonadectomy (GDX) in WT and CETP mice. Body weight was not different between GDX WT or GDX CETP male mice (Figure 5.5A). Plasma cholesterol was reduced in gonadectomized CETP males relative to gonadectomized WT males (Figure 5.5B). Whereas plasma TGs were increased over 70% in males with gonadal hormones (Figure 5.1C), gonadectomy completely abrogated the effect of CETP on plasma TGs (Figure 5.5C). In gonadectomized males, CETP expression reduced the cholesterol content of HDL fractions relative to gonadectomized WT males as measured by FPLC (Figure 5.5D), similar to males without gonadectomy surgery (Figure 5.1D). Gonadectomy completely abrogated the effect of CETP on TG concentration in VLDL fractions as measured by FPLC (Figure 5.5E). Thus, CETP expression reduces plasma cholesterol levels and HDL cholesterol content independent of sex-hormones in males. Furthermore, the hypertriglyceridemic effect of CETP did require gonadal hormones in males.

To confirm that gonadal hormones are required to impair postprandial TG excursion, TG clearance was measured in GDX WT and GDX CETP males. Following a 12hr fast, plasma TGs were measured after an oral bolus of olive oil (200 μl/mouse) in GDX WT and GDX CETP mice. Whereas CETP expression previously impaired postprandial TG excursion by over 80% in males without gonadectomy (Figure 5.2C-D), gonadectomy completely abrogated the effect of CETP on postprandial TG excursion (Figure 5.6A-B). Taken together, these data indicate that gonadal hormones are required for CETP to impair postprandial TG clearance and raise plasma TGs.

We demonstrated that the impaired postprandial TG clearance seen in CETP males corresponded with reduced expression in apolipoproteins and lipoprotein receptors that regulate TG uptake. We therefore examined plasma apolipoprotein levels and liver lipoprotein receptor expression to determine if gonadal hormones were required for CETP to alter apolipoprotein

levels and lipoprotein receptor expression. In the absence of gonadal hormones, CETP failed to increase plasma ApoB levels (Figure 5.7A-B). Additionally, CETP failed to reduce plasma ApoE levels in GDX CETP males relative to GDX WT males (Figure 5.7A-B). Furthermore, in the absence of gonadal hormones, CETP expression did not alter liver protein expression of LDLR or SRB1 (Figure 5.7C-D). We demonstrated that this reduction in LDLR corresponded with reduced liver cholesterol content in CETP males. In the absence of gonadal hormones, CETP failed to alter liver cholesterol (Figure 5.7E) or liver TG content (Figure 5.7F). Thus, gonadal hormones are required for CETP expression to reduce plasma apolipoprotein levels and reduce liver protein expression of lipoprotein uptake receptors. Taken together, these data demonstrate that gonadal hormones are required for CETP to impair postprandial TG clearance in males.

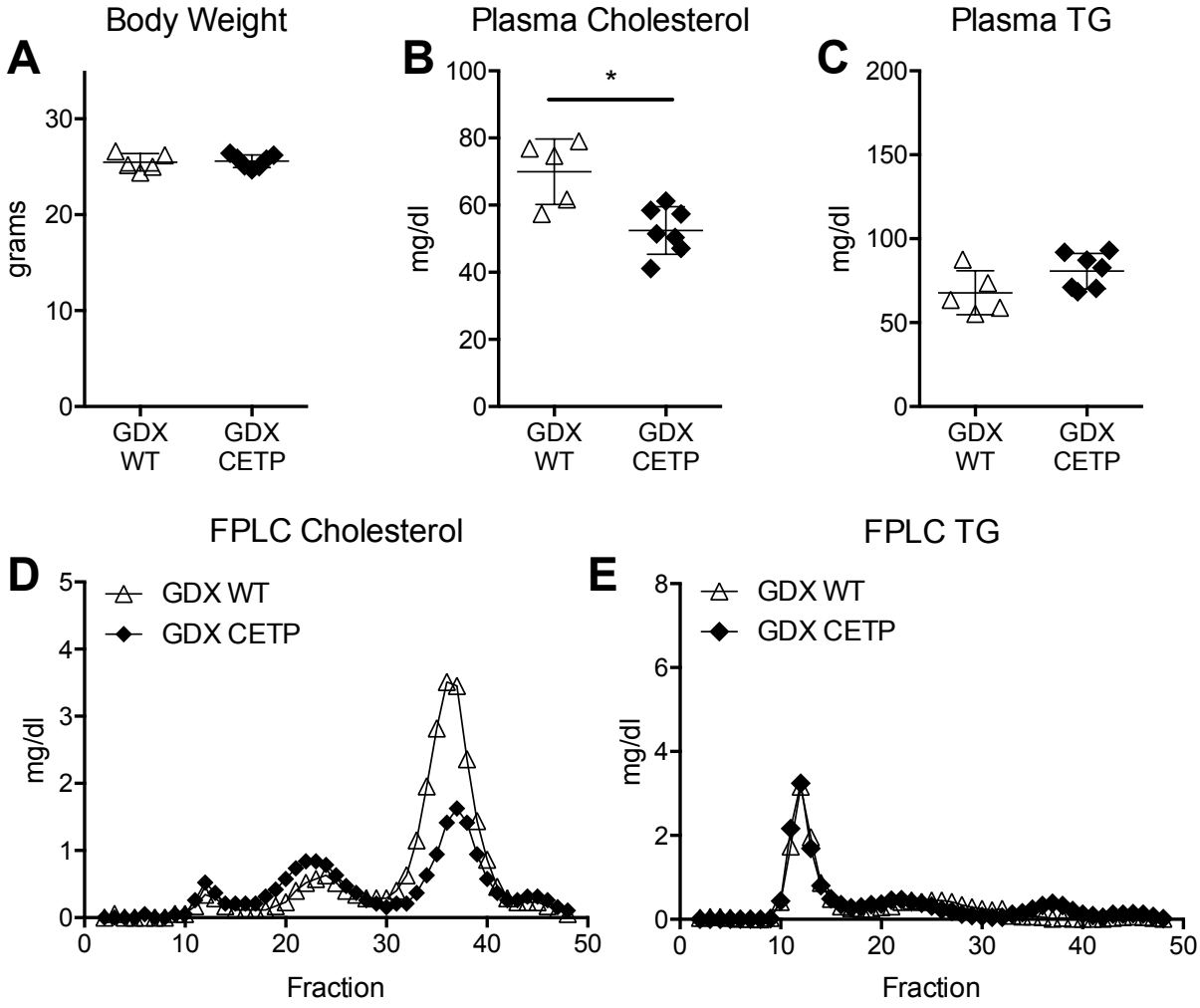

Figure 5.5. Gonadal hormones are required for CETP to raise plasma TGs in males. **A-C.** Body weight (A), plasma cholesterol (B), plasma TG (C) in gonadectomized (GDX) WT and CETP males. *P<0.05 (n=5-7/group) **D-E.** Cholesterol content (D) and TG content (E) of FPLC separation of pooled plasma lipoproteins. All data are summarized using mean ± SEM.

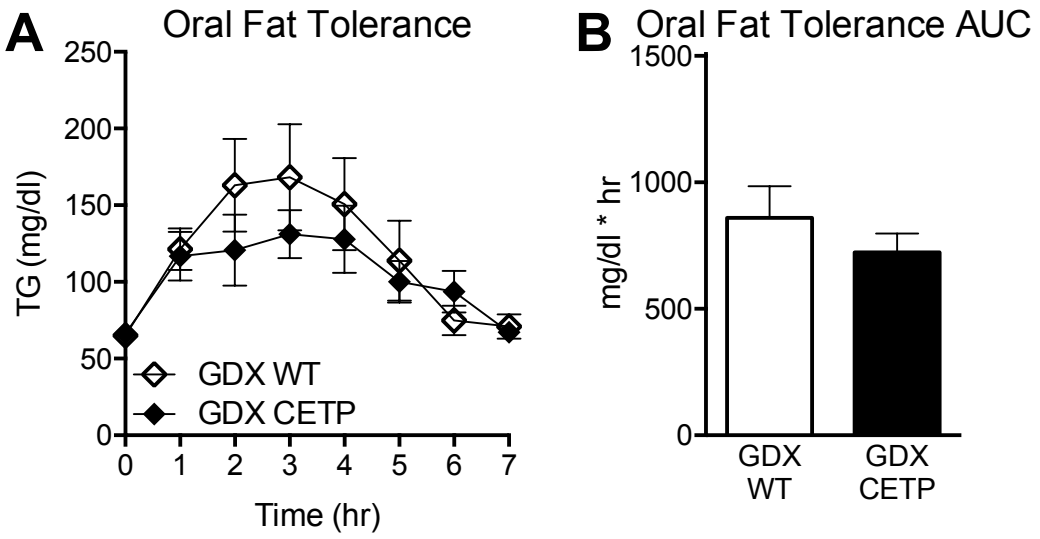

Figure 5.6. Gonadal hormones are required for CETP expression to impair postprandial TG clearance in males.

A. Plasma TGs following an oral bolus of olive oil (200 μl/mouse) in 12hr fasted GDX WT and GDX CETP males (n=6-7/group). **B.** Area under the curve (AUC) of oral fat tolerance. All data are summarized using mean ± SEM.

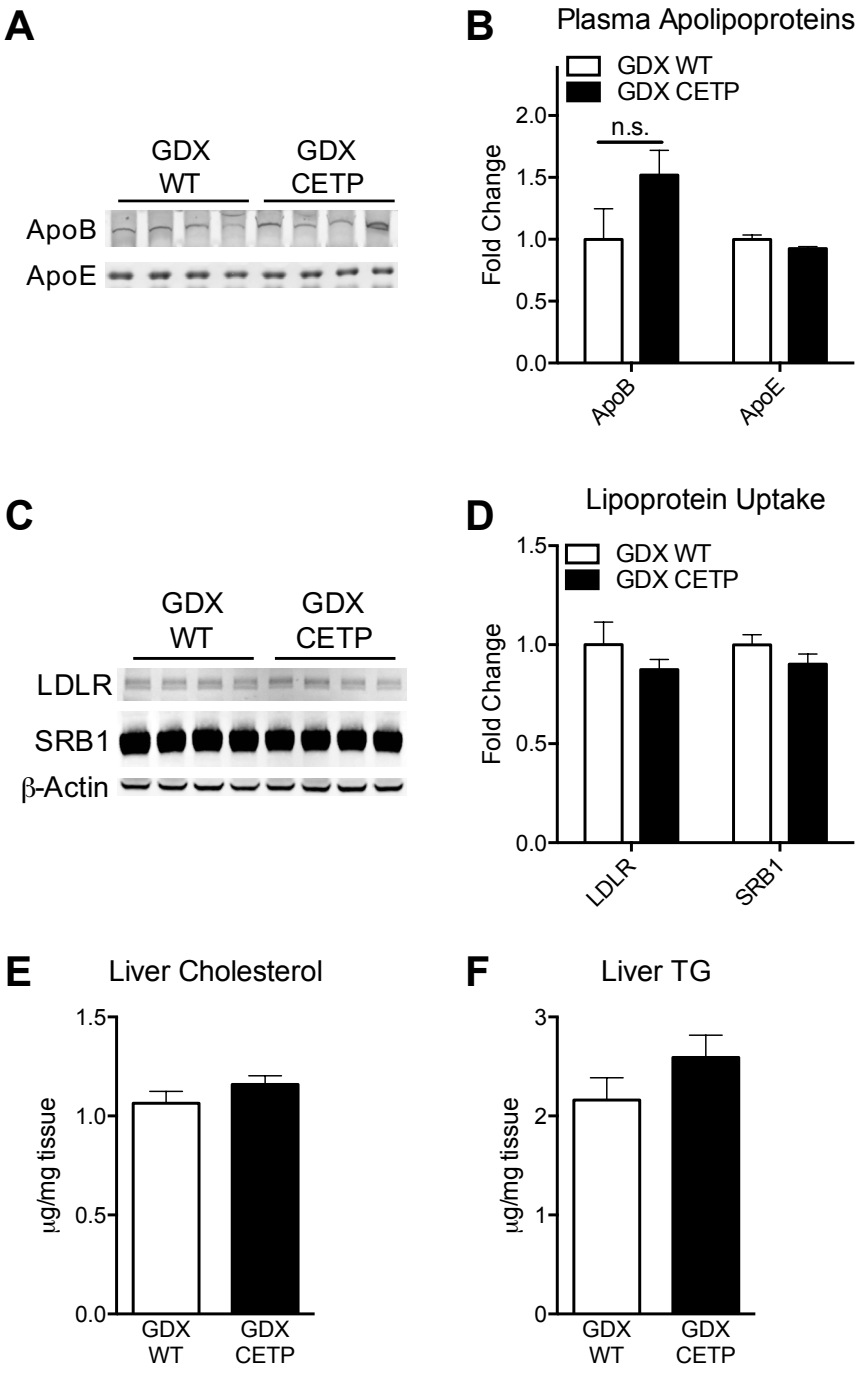

Figure 5.7. CETP does not alter TG clearance targets in the absence of gonadal hormones.

A-B. Western blot (A) and quantification (B) of plasma apolipoproteins from GDX WT and GDX CETP males (n=5-7/group). **C-D.** Western blot (C) and quantification (B) of liver lipoprotein receptors (n=5-7/group, normalized to b-actin). **E-F.** Liver cholesterol (E) and TG content (F) in GDX WT and GDX CETP males (n=5-7/group). All data are summarized using mean ± SEM.

CETP expression creates a gain-of-function response to gonadal hormones in males

Since CETP requires gonadal hormones to raise plasma TG levels, CETP may enhance normal responses to gonadal hormone function or CETP may mediate novel functions of gonadal hormones in male mice. If CETP enhanced normal gonadal function to raise plasma TGs, gonadectomy should result in a proportional decrease in plasma TGs in WT mice. If CETP created novel responses to gonadal hormones, gonadectomy should not alter plasma TGs in WT mice but should only alter plasma TGs in CETP mice. The effect of gonadectomy on plasma distribution of cholesterol and TG was compared in WT and CETP males. In WT and CETP males, gonadectomy did not alter the cholesterol content of lipoproteins as measured by FPLC (Figure 5.8A-B). Additionally, gonadectomy did not alter TG content of lipoproteins in WT males as measured by FPLC (Figure 5.8C). In CETP males, however, gonadectomy reduced VLDL-TG nearly 2-fold (Figure 5.8D). Since gonadectomy did not alter plasma lipoprotein TG content in WT males, but did alter plasma lipoprotein TG content in CETP males, we conclude that CETP did not enhance a normal effect of gonadal hormones. We conclude, rather, that CETP creates a gain-of-function response to gonadal hormones that results in elevated plasma TGs in males. This gain-of-function response to gonadal hormones does not involve alterations in testosterone availability since plasma testosterone levels were similar between WT and CETP males (Figure 5.8E) and GDX WT and GDX CETP males (Figure 5.8F). Thus, expression of CETP results in a novel signaling pathway dependent on gonadal hormones that impairs postprandial TG clearance in males.

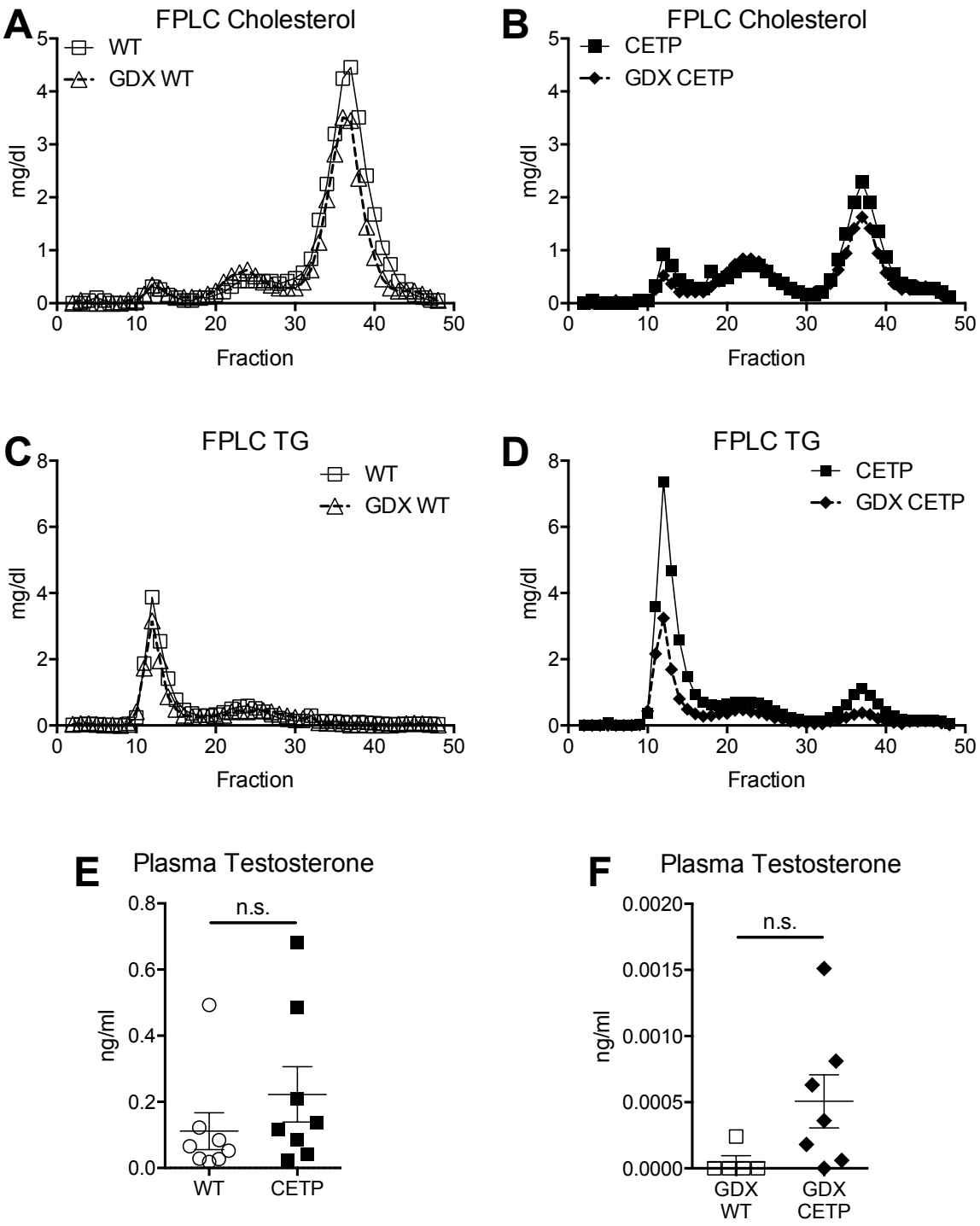

Figure 5.8. Gonadectomy alters TG distribution in lipoproteins only in CETP males. **A-B.** Cholesterol content in lipoproteins separated by FPLC in gonadectomized or intact WT (A) and CETP (B) males (pooled plasma from n=5-8/group). **C-D.** TG concentration in fractions separated by FPLC in gonadectomized or intact WT (C) and CETP (D) males. E-F. Plasma testosterone levels in WT and CETP males (A) or GDX WT and GDX CETP males (F) (n=5-8/group). All data are summarized using mean ± SEM.

Liver Estrogen Receptor α is not required for CETP to raise plasma TGs in males.

In males, the most abundant gonadal hormone in plasma is testosterone. Other androgens exist in plasma, including the proandrogen androstenedione and the more potent testosterone metabolite 5α-dihydrotestosterone (DHT). Although in significantly lower concentrations than females, males have detectable levels of estrogen in plasma. Many effects typically attributed to testosterone (sex drive, erectile function) require aromatization to estrogen (311), suggesting that estrogen receptors may mediate some of the functions of testosterone. Additionally, testosterone has been shown to have beneficial effects on liver lipid metabolism and atherosclerosis in mouse models lacking the androgen receptor (312, 313), which indicates that testosterone to aromatization to estrogen may not be an important avenue by which gonadal hormones alter lipid metabolism in males. Estrogen receptor α (ERα) is the predominant liver estrogen receptor (287) and has been shown to play an important role in liver and plasma TG metabolism (110, 286). To determine if liver ERα is required for CETP to raise plasma TGs in males, we bred CETP onto a congenic strain with a liver-specific deletion of ERα (LKO-ERα). Body weight was not different in LKO-ERα or LKO-ERα CETP males (Figure 5.9A). Despite deletion of liver ERα, CETP expression still reduced plasma cholesterol levels (Figure 5.9B) and increased plasma TGs (Figure 5.9C) in males. Correspondingly, cholesterol concentration of HDL fractions was reduced in LKO-ERα CETP males relative to LKO-ERα males (Figure 5.9D). Additionally, TG concentration in VLDL fractions was greatly increased in LKO-ERα CETP relative to LKO-ERα males (Figure 5.9E). Thus, CETP likely does not utilize gonadal hormone action on liver ERα to increase plasma TG levels in males.

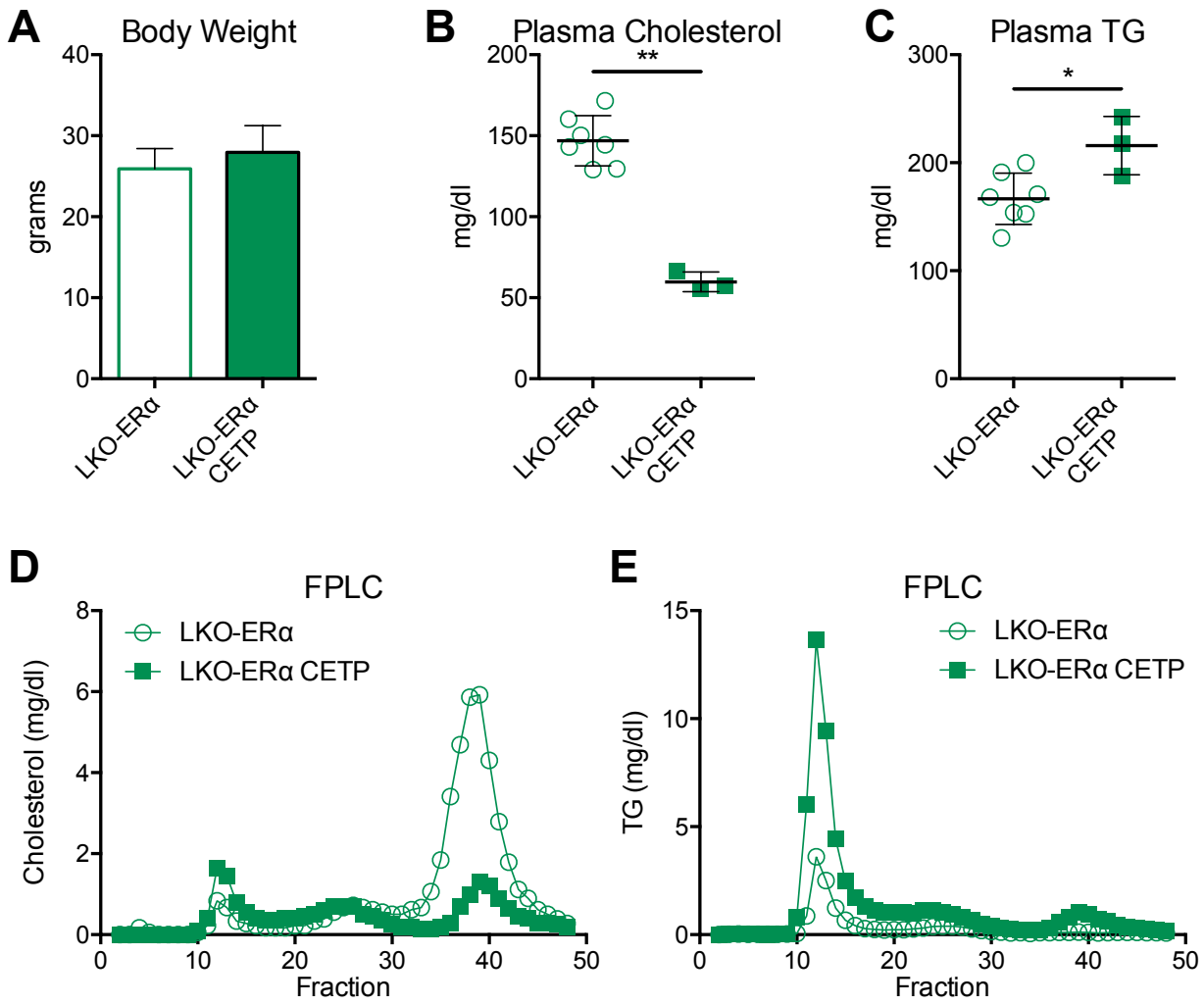

Figure 5.9. Liver ERα is not required for CETP to raise plasma TGs in males.
A-C. Body weight (A), plasma cholesterol (B), and plasma TGs (C) in male mice with liver-specific knockout of ERα (LKO-ERα) and CETP mice with liver-specific knockout of ERα (LKO-ERα CETP). **D-E.** Cholesterol content (D) and TG content (E) of FPLC fractions from pooled plasma (n=3-7/group). All data are summarized using mean ± SEM.

Discussion

Our previous data demonstrated a role for CETP in TG metabolism in females, especially with estrogen treatment (264). The role of CETP in regulating TG metabolism in males, however, is unknown. Here we demonstrate that CETP alters TG metabolism in male mice *in-vivo*. Expression of CETP raised plasma TGs by impairing postprandial TG clearance in males without altering TG production. This impaired TG clearance corresponded with both lower levels of plasma apoE, and also, lower expression of liver LDLR and SRB1, which regulate lipoprotein uptake. This reduction in LDLR expression corresponded with reduced liver cholesterol content in CETP males. Since we previously showed that CETP required estrogen to raise plasma TGs in females, we determined whether gonadal hormones were required for CETP to impair TG clearance in males. Removal of gonadal hormones completely attenuated the effects of CETP on plasma TG levels, postprandial TG clearance, and liver lipoprotein receptor expression. Thus, CETP contributes to increased plasma TGs in males through a mechanism involving gonadal hormone action to reduce liver lipoprotein receptor expression.

Although we demonstrate that CETP expression contributes to increased plasma TGs in males and females, the mechanisms by which CETP raises plasma TGs differs in males compared to females. In both male and female mice, CETP expression results in increased plasma TGs and increased VLDL-TG. This effect required sex hormones in males and females. In females, estrogen was required for CETP to increase VLDL-TG production. In males, though, gonadal hormones were required for CETP to impair TG clearance. Female CETP mice also had impaired TG clearance relative to WT females, but this effect was independent of estrogen treatment. Thus, while CETP seems to impair TG clearance in both males and females, male CETP mice require sex hormones for this effect, while female CETP mice did not. In males, the impaired TG clearance seems to be due to reduced LDLR protein expression, an effect lost with the removal of gonadal hormones. The mechanism for impaired TG clearance in CETP females

remains unknown. In addition to the effects on plasma TGs, CETP expression also alters liver lipid metabolism. In females, CETP expression reduced liver TG content, an effect that required liver ERα. In males, CETP did not alter liver TG content, perhaps due to low liver ERα expression. In contrast to females, CETP expression reduced liver cholesterol content in males, whereas CETP did not alter liver cholesterol content in any female mice (Chapters 3 and 4). The reduced liver cholesterol content seen in CETP males was likely due to reduced liver LDLR expression. CETP failed to reduce liver cholesterol content in males in the absence of gonadal hormones, likely because LDLR expression was comparable to control WT males. Thus, CETP expression alters plasma TG levels in both males and females, but by different mechanisms that require sex hormones.

Although we demonstrate that gonadal hormones are required for CETP to impede TG clearance in males, the mechanism by which CETP alters gonadal hormone signaling remains to be determined. CETP did not alter plasma testosterone levels in either intact or gonadectomized males (Figure 5.8E-F). Furthermore, CETP did not enhance a “normal” effect of gonadal hormones on TG metabolism since plasma TG levels did not change with gonadectomy in WT males. Additionally, aromatization of testosterone to estrogen likely does not contribute to the effect of CETP on TG metabolism since CETP expression increased plasma TGs and VLDL-TG in the absence of liver ERα. Therefore, CETP may alter androgen receptor (AR) function in males, similar to the requirement of liver ERα in females (Chapter 4). In females, however, ERα was only required for certain effects of CETP action on TG homeostasis. Additionally, testosterone is not known to regulate LDLR in men or in human cell lines *in-vitro*. It is plausible, however, that testosterone may regulate LDLR in the presence of CETP only in mice. It is also possible that gonadal hormone action in other tissues may regulate liver LDLR expression indirectly, perhaps through adipokine or other endocrine pathways.

Further work will be needed to determine if liver AR is required for CETP to reduce LDLR expression and impair TG clearance.

The work presented here suggests that CETP may be pro-atherogenic in males since CETP expression impairs TG clearance. Impaired TG clearance is an important risk factor for atherosclerosis in men (154-157, 159, 304). The role of CETP in atherosclerosis in humans is uncertain. While some studies suggest a positive correlation between CETP activity (202-205) and risk of cardiovascular disease, other studies suggest a neutral (314-316) or inverse (203, 206-211) correlation with cardiovascular disease. Interestingly, CETP activity seems to increase risk of cardiovascular disease when TG levels are high (205). When TG levels were below the median, the association between CETP and cardiovascular disease was lost. This may suggest that CETP activity is pro-atherogenic only in the context of hypertriglyceridemia. This is likely confounded, however, by hyperinsulinemia and obesity, two factors known to alter CETP activity and also increase plasma TGs (317, 318).

While data presented here support the potential proatherogenicity of CETP in males, the contribution of CETP to cardiovascular disease may be diminished in the setting of concurrent statin treatment. Our data demonstrate that CETP impairs TG clearance in male mice, which corresponded with reduced liver LDLR expression. Statin therapy, the mainstay of modern cardiovascular disease treatment and prevention, increases LDLR expression (319). This increased LDLR expression contributes to reducing LDL cholesterol levels. This increased LDLR expression also increases TG clearance, especially in hypertriglyceridemic patients (320). CETP inhibitors were developed under the assumption that CETP is proatherogenic. CETP inhibitors reduce plasma TG levels by increasing apoB and TG clearance (245-247). The effect of anacetrapib on reducing plasma TGs seems to be negated by concurrent treatment with a statin. Although certain beneficial effects of CETP inhibitors on lipoprotein kinetics persist with concurrent statin therapy, it remains to be determined whether CETP inhibitors will reduce cardiovascular disease outcomes, especially in the setting of concurrent statin therapy. CETP

inhibitors may provide an alternative to reduce TGs and LDL cholesterol in the statin-intolerant patient population. Thus, future work aimed at understanding how CETP alters TG metabolism may lead to more targeted therapies that reduce risk of cardiovascular disease.

CHAPTER VI

LOW-DENSITY LIPOPROTEIN RECEPTOR IS REQUIRED FOR CETP TO ALTER TG METABOLISM IN BOTH MALE AND FEMALE MICE

Abstract

Elevated plasma triglycerides (TGs) are an important risk factor for cardiovascular disease. Impaired TG clearance is an important mechanism by which elevated plasma TGs increase risk of cardiovascular disease. We previously demonstrated that expression of Cholesteryl Ester Transfer Protein (CETP) in mice, which naturally lack CETP, results in increased plasma TGs in male and female mice but by different mechanisms. In females, estrogen was required for CETP to raise Very Low Density Lipoprotein (VLDL) TG production. In males, however, gonadal hormones were required for CETP to impair TG clearance. A unifying mechanism that explains how CETP alters TG metabolism in both males and females is currently lacking. Low Density Lipoprotein Receptor (LDLR) has a well-established role in regulating TG clearance. LDLR is also known to regulate VLDL-TG production. Therefore, LDLR was an attractive molecular target that may mediate the effects of CETP on TG metabolism in both males and female mice. Here, we demonstrate that LDLR was required for CETP to increase TG production in response to estrogen in females and for CETP to impair TG clearance in males. These results indicate that delivery of lipid to the liver by CETP through LDLR may alter liver TG metabolism. Further understanding of the role of LDLR in mediating the effects of CETP on TG metabolism may lead to discovery of novel targets regulating both TG production and clearance.

Introduction

Increased plasma TGs are an important risk factor for cardiovascular disease (147, 149-151). Both overproduction and impaired clearance of plasma TGs contribute to elevated plasma TGs. While fasting plasma TGs seems to be an important risk factor for cardiovascular disease only in women, impaired postprandial TG clearance increases risk of cardiovascular disease in both men and women (8, 152, 154-156, 158, 159, 304). Understanding pathways that regulate TG clearance may lead to novel therapies that reduce risk of cardiovascular disease.

We previously demonstrated that expression of CETP acts to raise plasma TG levels in females and males, but by different mechanisms (Chapters 3 and 4) (264). In females, CETP expression increases TGs by increasing VLDL production in response to estrogen treatment. In males, CETP expression increased plasma TGs by impairing postprandial TG clearance (Chapter 5). In both males and females, the ability of CETP to raise plasma TGs was dependent on sex hormones. In females, we previously showed that CETP signals *via* two distinct networks to govern different aspects TG metabolism (Chapter 4). It remains to be determined if a single molecular target is responsible for all of the effects of CETP on TG metabolism. Furthermore, how CETP, a secreted plasma protein, alters intracellular nuclear signaling to govern TG metabolism remains an unanswered question. One potential way CETP may alter TG homeostasis is by altering delivery of TG to a cell surface receptor that regulates both TG clearance and TG production. One such potential mediator of CETP action on TG metabolism is the Low-Density Lipoprotein Receptor (LDLR).

LDLR is a cell surface transmembrane receptor that binds to apolipoprotein B (apoB) or apolipoprotein E (apoE) on chylomicrons, VLDL or LDL (321). Upon ligand binding to LDLR, holoparticle lipoprotein uptake *via* clathrin-mediated endocytosis results in cellular uptake of lipoproteins (322). LDLR has a well-established role in TG clearance (137, 138, 323-325). In addition to regulating TG clearance, LDLR has also been shown to regulate TG production *in-*

vitro (324, 326), in mouse models *in-vivo* (327-329), and in humans with familial hypercholesterolemia (323, 325, 330-332), a genetic disease characterized by deficiency of the LDLR. Thus, LDLR regulates both TG clearance and production, and represents a unique molecular target that may mediate the effects of CETP on TG metabolism in both male and female mice. Here, we explore the role of LDLR in regulating TG metabolism in male and female mice expressing CETP. We postulated that expression of CETP in mice allows for a novel route of lipid delivery to the liver, which has important implications for TG metabolism. Using mice with genetic deletion of LDLR, we demonstrate that LDLR is required for CETP to increase VLDL production in response to estrogen in females and that LDLR is required for CETP to impair clearance of postprandial TGs in males. Thus, LDLR is a major upstream determinant of the ability of CETP to impact TG homeostasis *in-vivo*.

Results

Low Density Lipoprotein Receptor is Required for Estrogen to Raise Plasma TGs in Response to Estrogen in CETP Females.

To determine if the ability of CETP to alter liver and plasma TG metabolism requires LDLR, CETP mice were bred with mice harboring a global knockout of LDLR ($LDLR^{-/-}$). Females were ovariectomized to reduce variability in estrus cycling and to remove the contribution of endogenous hormones. Following 6-7 days of recovery, female mice were then treated with vehicle (sesame oil) or estrogen (1μg/g, 17β-estradiol-3-benzoate) to determine if CETP required LDLR to alter liver and plasma TG metabolism in response to estrogen. Estrogen treatment did not alter body weight in $LDLR^{-/-}$ or $LDLR^{-/-}$CETP female mice (Figure 6.1A). Estrogen raised uterine weight equally in both $LDLR^{-/-}$ and $LDLR^{-/-}$CETP females (Figure 6.1B), indicating a similar response to estrogen treatment. Plasma cholesterol was unchanged by estrogen in $LDLR^{-/-}$ and $LDLR^{-/-}$CETP females (Figure 6.1C). Estrogen treatment modestly reduced plasma TGs to a similar degree in both $LDLR^{-/-}$ and $LDLR^{-/-}$CETP females (Figure 6.1D). Importantly, estrogen failed to raise plasma TGs in response to estrogen in CETP females lacking LDLR. As expected, deletion of LDLR increased LDL and VLDL levels as seen by FPLC separation of plasma lipoproteins (Figure 6.1E-H). Estrogen treatment did not significantly affect cholesterol distribution in FPLC fractionation of plasma lipoproteins in either $LDLR^{-/-}$ or $LDLR^{-/-}$CETP (Figure 6.1E-F). Previously, estrogen treatment nearly doubled the TG concentration in VLDL fractions of plasma from CETP mice (Figure 3.2F). In the absence of LDLR, however, estrogen treatment did not increase the TG concentration in VLDL or LDL fractions in $LDLR^{-/-}$CETP females (Figure 6.1H). Thus, LDLR is required for estrogen to raise plasma TGs and increase the TG concentration in VLDL in females expressing CETP.

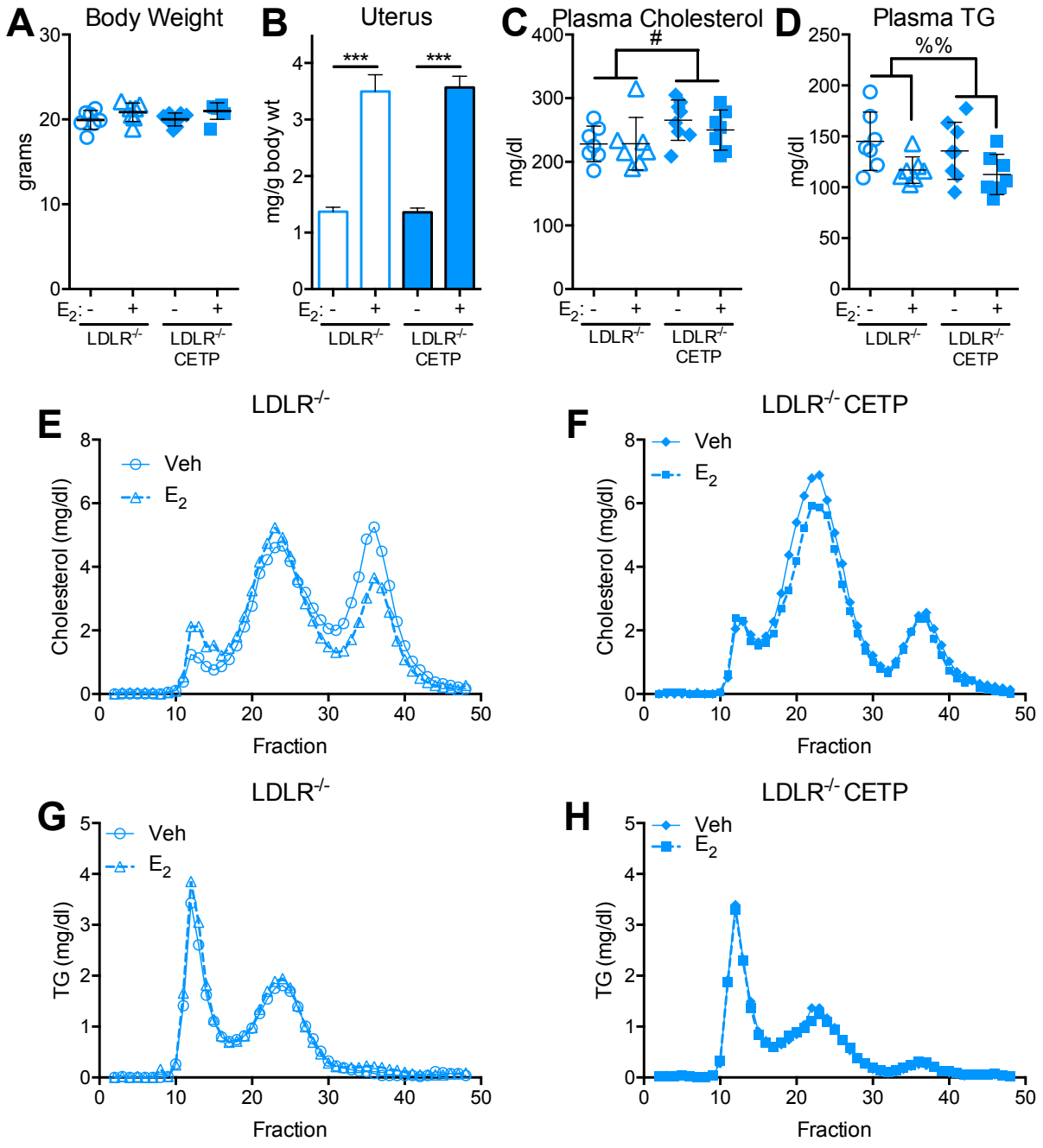

Figure 6.1. LDLR is required for CETP to raise plasma TGs in response to estrogen in females.

A-D. Body weight (A), uterine weight (B), plasma cholesterol (C), plasma TG (D) of LDLR$^{-/-}$ and LDLR$^{-/-}$CETP females treated with vehicle (-) or estrogen (+). ***P<0.001 (ANOVA), $^{\#}$P<0.05 for genotype effect, $^{\%\%}$P<0.01 for estrogen effect (2-way ANOVA), n=7-8/group. **E-H.** Fast performance liquid chromatography (FPLC) separation of plasma lipoproteins from pooled plasma from LDLR$^{-/-}$ and LDLR$^{-/-}$CETP females treated with vehicle (Veh) or estrogen (E_2). Cholesterol content of FPLC fractions from LDLR$^{-/-}$ and LDLR$^{-/-}$ CETP females. **G-H.** TG concentration of FPLC fractions from LDLR-/- (G) and LDLR$^{-/-}$CETP (H) females. All data are summarized using mean ± SEM.

Low Density Lipoprotein Receptor is Required for CETP to Raise TG Production in Response to Estrogen in Females.

To confirm that LDLR is required for CETP to raise VLDL production in response to estrogen, we measured TG production in LDLR$^{-/-}$ and LDLR$^{-/-}$CETP females with and without estrogen treatment. Estrogen treatment did not alter TG production in LDLR$^{-/-}$ females (Figure 6.2A). In the absence of LDLR, estrogen treatment failed to increase TG production in LDLR$^{-/-}$ CETP females (Figure 6.2B). We previously showed that estrogen induced expression of VLDL synthesis and assembly targets in the liver. In particular, estrogen seemed to induce expression and activity of PDI. In the absence of LDLR, estrogen failed to increase liver PDI activity in LDLR$^{-/-}$CETP females (Figure 6.2C). Furthermore, estrogen failed to increase expression of mRNA for PDI isoforms or other genes involved in VLDL synthesis and assembly (Figure 6.2D). In fact, mRNA levels of PDI isoforms *P4hb* and *Pdia3* were lower in LDLR$^{-/-}$CETP females relative to LDLR$^{-/-}$ females (Figure 6.2D). Whereas estrogen previously did not significantly alter mRNA levels of genes involved in VLDL synthesis and assembly in WT females (Figure 3.3G), estrogen treatment significantly altered the mRNA expression a number of these target genes in females lacking LDLR (*Apob, Mttp, Pdia3, Pdia4, Arf1, Sort1*, Figure 6.2D). Deletion of LDLR, therefore, seems to generate a novel effect of estrogen on genes involved in VLDL synthesis and assembly. The net effect of these changes, however, did not alter VLDL-TG production. These results confirm that LDLR is required for CETP expression to increase VLDL production and to increase mRNA levels and protein activity of genes involved in VLDL synthesis and assembly.

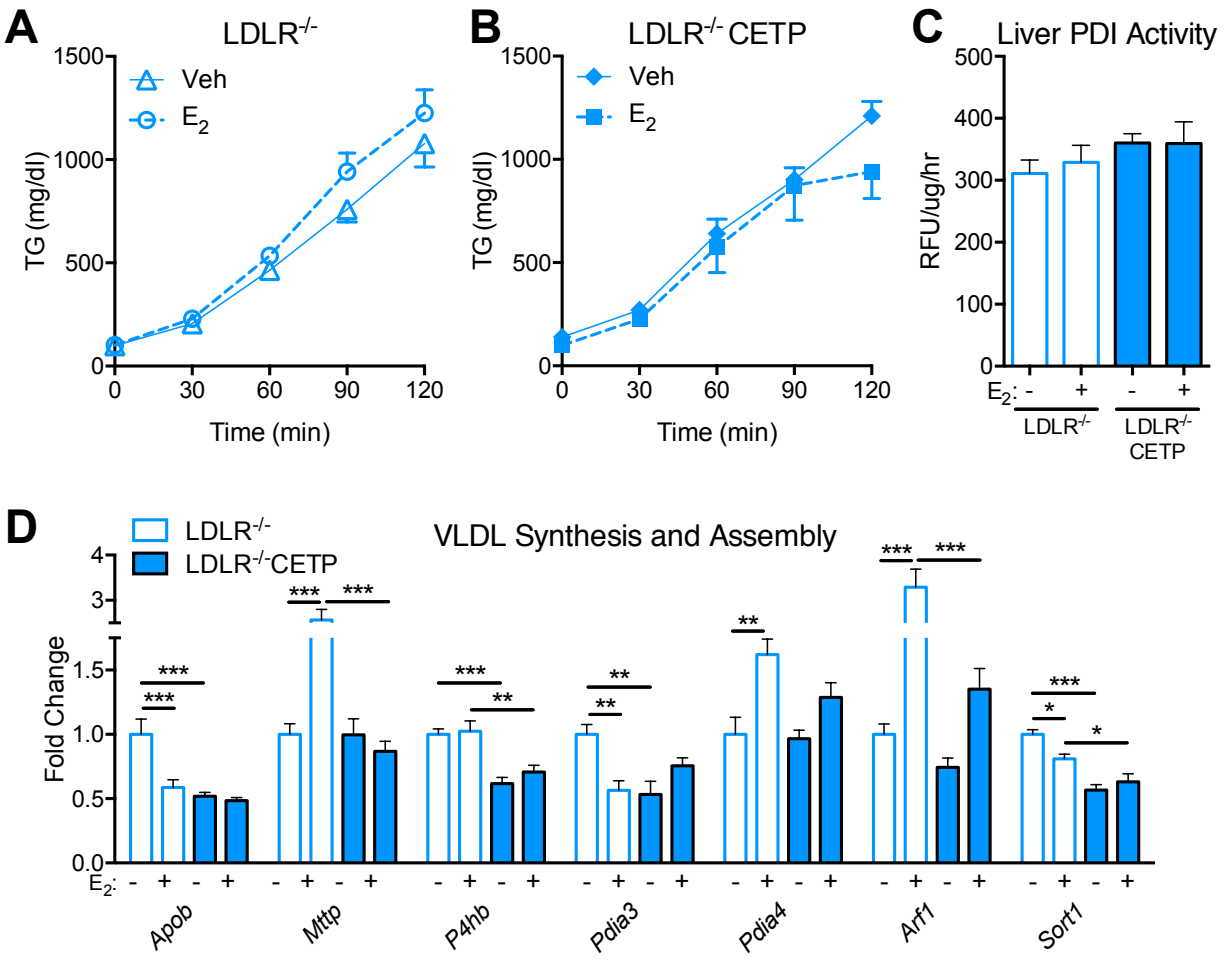

Figure 6.2. LDLR is required for CETP to raise VLDL production in response to estrogen. **A-B.** TG production after intraperitoneal injection of poloxamer 407 (1000 mg/kg) in 3hr fasted LDLR$^{-/-}$ and LDLR$^{-/-}$CETP females treated with vehicle (sesame oil) or estrogen (E_2). **C.** Liver PDI activity in LDLR$^{-/-}$ and LDLR$^{-/-}$CETP females treated with vehicle (-) or estrogen (+) (n=7-8/group). **D.** Liver mRNA expression of genes involved in VLDL synthesis and assembly in in LDLR$^{-/-}$ and LDLR$^{-/-}$CETP females treated with vehicle (-) or estrogen (+) (*P<0.05, **P<0.01, ***P<0.001, ANOVA, n=7-8/group). All data are summarized using mean ± SEM.

Low Density Lipoprotein Receptor is Partially Required for CETP to Lower Liver TG Content in Females.

Since LDLR was required for CETP to raise VLDL production in response to estrogen, we next determined if LDLR was required for the ability of CETP to alter liver TG content. Expression of CETP in the absence of LDLR did not alter liver cholesterol content (Figure 6.3A). Estrogen treatment increased liver cholesterol content by ~15% in $LDLR^{-/-}$ females (Figure 6.3A). Estrogen treatment did not previously alter liver cholesterol content in either WT or CETP females (Figure 3.6B). Deletion of LDLR resulted in a dramatic increase in liver TG content relative to WT females (9.73 ± 2.02 vs. 5.82 ± 0.81 mg/g liver, $LDLR^{-/-}$ veh vs. WT veh, $P<0.001$, Figure 6.3B, Figure 3.6A). In the absence of LDLR, CETP expression reduced liver TG content by 25% relative to control females (7.35 ± 1.11 vs 9.73 ± 2.02 mg/g liver, $LDLR^{-/-}$CETP veh vs $LDLR^{-/-}$ veh, $P<0.05$, Figure 6.3B). Previously, we demonstrated that CETP expression reduces liver TG content by 60% relative to WT controls (Figure 3.6A). Although CETP reduced liver TG content in the absence of LDLR, the effect of CETP on reducing liver TG content was greatly diminished with deletion of LDLR. Thus, LDLR is required for at least half of the effect of CETP expression on reducing liver TG content. Estrogen treatment reduced liver TG content to similar levels in $LDLR^{-/-}$ and $LDLR^{-/-}$CETP females (Figure 6.3B).

We previously demonstrated that the ability of CETP expression to reduce liver TG content correlated with increases in liver β-oxidation gene expression and plasma metabolite levels (Figure 3.6C-D). To determine if CETP could reduce liver TG content in the absence of LDLR by a similar mechanism, we analyzed liver mRNA levels of β-oxidation target genes. Surprisingly, CETP expression resulted in reduced liver mRNA levels of β-oxidation targets (*Ppara, Cpt2, Acox1*, Figure 6.3C). Estrogen treatment resulted in reduced mRNA levels of β-oxidation target

genes in LDLR$^{-/-}$ females (*Ppara, Cpt1a, Cpt2, Acox1, Acadm*, Figure 6.3C). This reduction mRNA level of β-oxidation target genes was unexpected given the low levels of liver TG content seen in estrogen treated LDLR$^{-/-}$ females (Figure 6.3B). The mRNA levels were measured at 24 hr after estrogen treatment. One possible explanation for the low levels of mRNA expression of β-oxidation target genes could be a counter-regulatory response to a transient induction of expression by estrogen prior to the time of measurement. Further understanding of the kinetic response of β-oxidation target gene expression to estrogen treatment may explain the discrepancy between low liver TG levels and low liver β-oxidation gene expression. Although CETP expression resulted in a reduction in β-oxidation target gene expression despite lower liver TG content, CETP expression was still able to disrupt estrogen-mediated regulation of β-oxidation targets in the absence of LDLR. Expression of CETP resulted in a blunted response to estrogen for certain genes in LDLR$^{-/-}$CETP females relative to LDLR$^{-/-}$ females (*Ppara, Cpt2, Acox1, Acadm,* Figure 6.3C). Thus, despite the absence of LDLR, CETP is still able to alter estrogen action on target gene expression in the liver. Additionally, deletion of LDLR seems to diminish the ability of CETP expression to reduce liver TG content and increase β-oxidation target gene expression. Therefore, the effect of CETP expression on reducing liver TG content in the absence of LDLR likely occurs by a novel mechanism than in the presence of LDLR. Taken together, these results indicate that the ability of CETP to alter TG metabolism are largely dependent on LDLR expression.

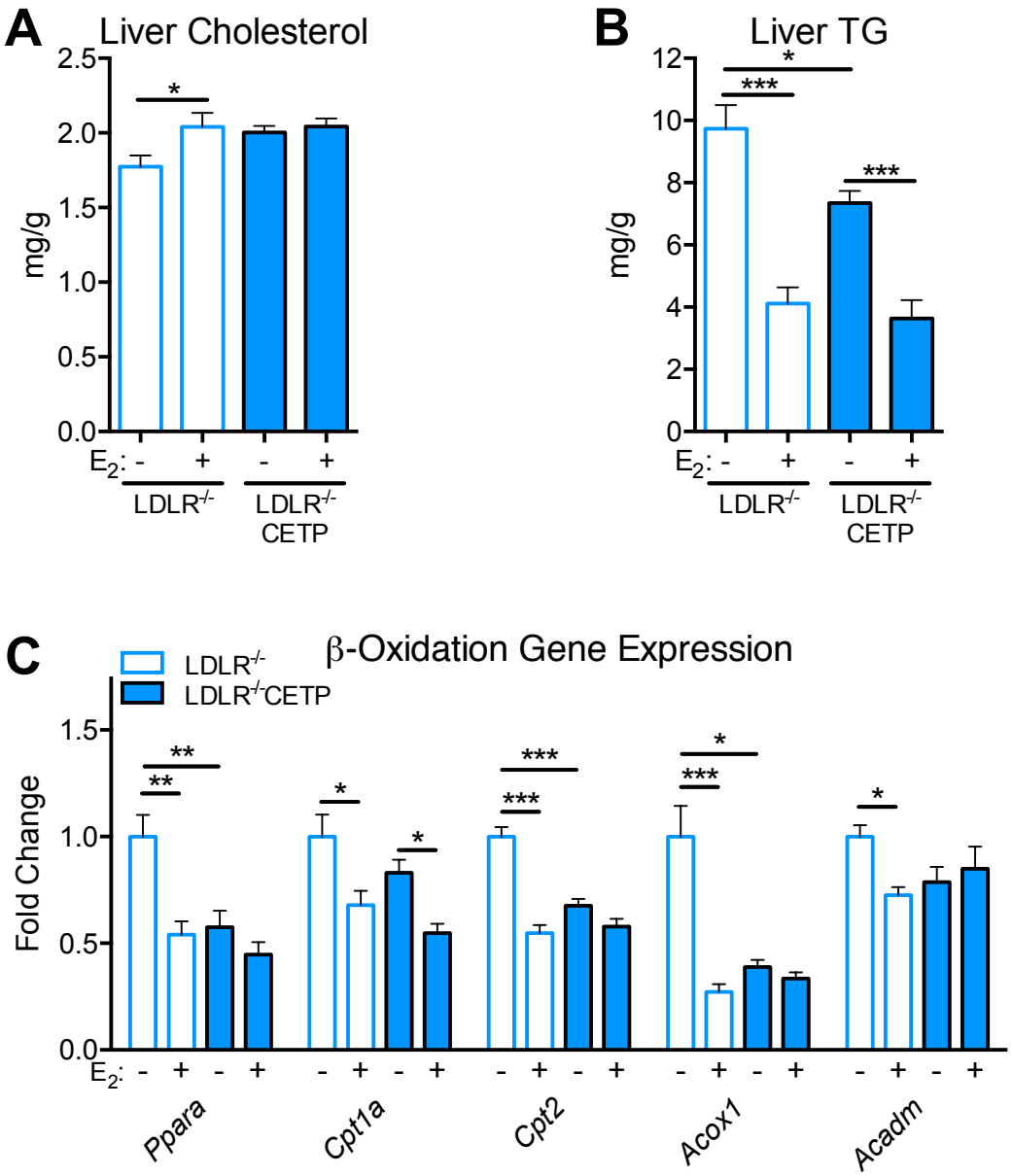

Figure 6.3. LDLR is partly required for CETP expression to reduce liver TG content. **A-B.** Liver cholesterol (A) and TG (B) content in LDLR$^{-/-}$ and LDLR$^{-/-}$CETP females treated with vehicle (-) or E_2 (+) (*P<0.05, ***P<0.001, ANOVA, n=7-8/group). **C.** Liver mRNA expression of genex involved in β-oxidation (*P<0.05, **P<0.01, ***P<0.001, ANOVA, n=7-8/group). All data are summarized using mean ± SEM.

Low Density Lipoprotein Receptor is Required for CETP to Raise Plasma TGs in Males.

Since we show that LDLR is largely required for the effects that CETP has on TG metabolism in female mice, we hypothesized that LDLR might also be required for CETP to alter TG metabolism in males. To test the hypothesis that LDLR was required for CETP to alter TG metabolism in males, we utilized $LDLR^{-/-}$ and $LDLR^{-/-}$CETP males from the genetic breeding described above. CETP did not alter body weight in $LDLR^{-/-}$ and $LDLR^{-/-}$CETP males (Figure 6.4A). In the absence of LDLR, CETP did not alter plasma cholesterol levels (Figure 6.4B). Additionally, in the absence of LDLR, CETP did not raise plasma TG levels relative to control males (Figure 6.4C). Analysis of plasma lipoprotein distribution as measured by FPLC revealed that deletion of LDLR had the expected effect of increasing LDL and VLDL levels relative to WT mice (Figure 6.4D-E). In the absence of LDLR, CETP expression reduced HDL cholesterol, but also increased LDL and VLDL cholesterol relative to $LDLR^{-/-}$ males (Figure 6.4D). This resulted in a net neutral effect on total plasma cholesterol levels. In the absence of LDLR, CETP expression did not increase TG concentration in VLDL, LDL or HDL fractions (Figure 6.4E). CETP expression resulted in slightly increased lipoprotein size as indicated by the left-ward shift in VLDL, LDL and HDL peaks in $LDLR^{-/-}$CETP males relative to $LDLR^{-/-}$ males (Figure 6.4D-E). Thus, LDLR is required for CETP to raise plasma TGs in males.

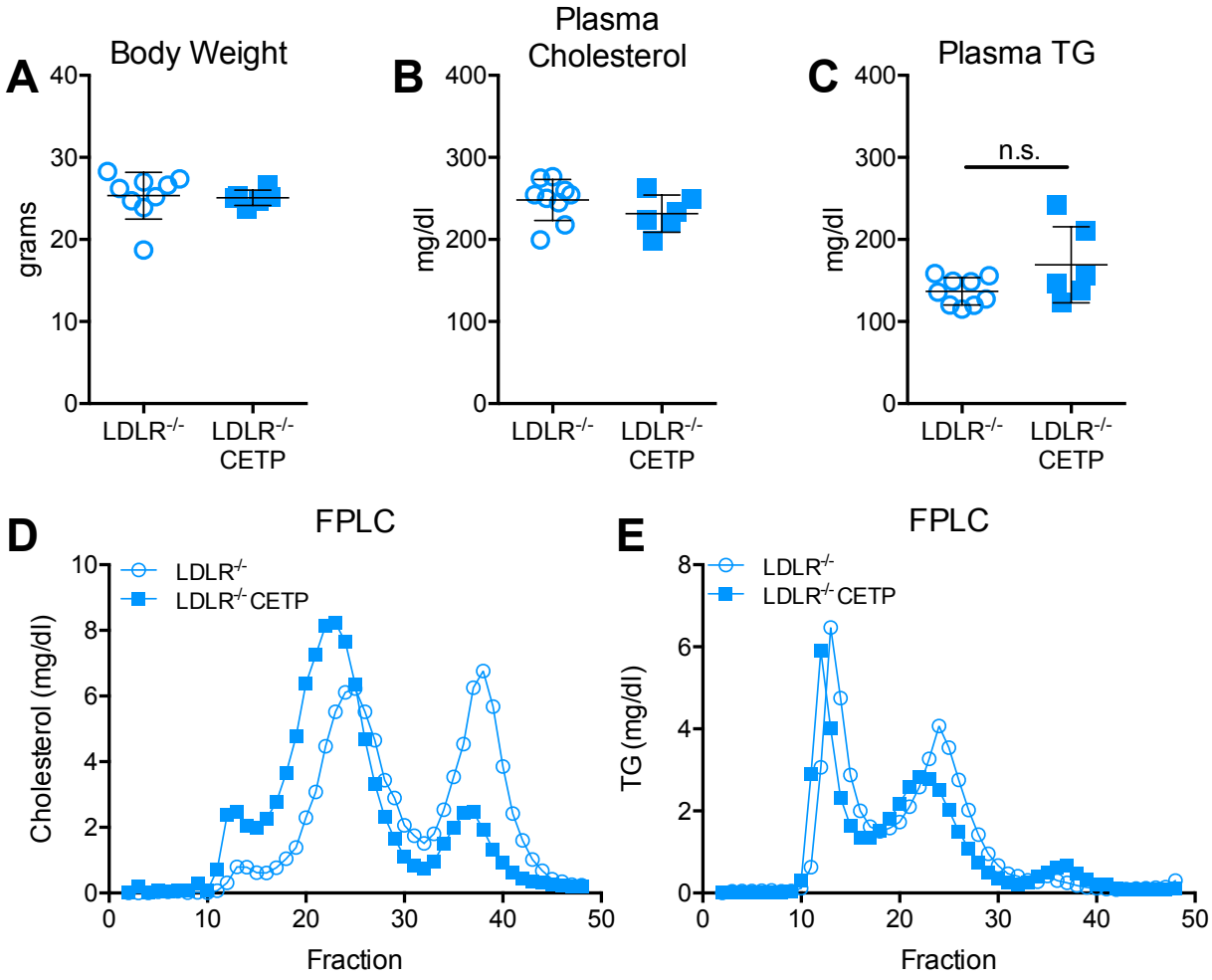

Figure 6.4. LDLR is required for CETP to raise plasma TGs in males.
A-C. Body weight (A), plasma cholesterol (B), and plasma TG (C) in LDLR$^{-/-}$ and LDLR$^{-/-}$CETP males (n=7-9/group). **D-E.** Cholesterol content (D) and TG content (E) of fractions of plasma separated by FPLC (pooled plasma from n=7-9/group). All data are summarized using mean ± SEM.

Low Density Lipoprotein Receptor is Required for CETP to Delay Postprandial TG Clearance in Males.

Since LDLR is required for CETP to raise plasma TG levels in males, we next determined if LDLR was required for CETP to impair postprandial TG excursion in males. We previously demonstrated that CETP expression in males resulted in a greater postprandial TG excursion following an oral bolus of fat relative to WT males (Figure 5.2C-D). To determine if LDLR was required for this effect in males, we measured postprandial TG excursion in response to an oral fat bolus in $LDLR^{-/-}$ and $LDLR^{-/-}$CETP males. In the absence of LDLR, CETP expression failed to impair postprandial TG excursion in males (Figure 6.5A). TG clearance, as indicated by the area under the curve, was not impaired in $LDLR^{-/-}$CETP males relative to $LDLR^{-/-}$ males (Figure 6.5B). Our previous data in males suggested that CETP expression resulted in reduced expression of apolipoprotein ligands that promote TG uptake (ApoE) and reduced expression of receptors that regulate lipoprotein uptake (LDLR, SRB1). To determine if LDLR was required for CETP to alter apolipoprotein expression or expression of other lipoprotein uptake receptors, we examined plasma and liver targets involved in TG uptake. CETP expression resulted in increased plasma protein levels of ApoB and decreased plasma protein levels of ApoE, ApoA1 and ApoC1 in $LDLR^{-/-}$CETP males relative to $LDLR^{-/-}$ males (Figure 6.5C-D). The reduction in ApoE and reduction on ApoC1 may result in a net neutral effect on plasma TG clearance. CETP expression failed to alter expression of SRB1 in the absence of LDLR (Figure 6.5E-F). Overall, CETP expression was able to alter plasma lipoprotein levels in the absence of LDLR, highlighting the importance of LDLR in mediating the ability of CETP to impair postprandial TG clearance. Thus, LDLR is required for CETP to raise plasma TG levels and impair postprandial TG clearance. LDLR was not required, however, for the ability of CETP to alter plasma apolipoprotein levels.

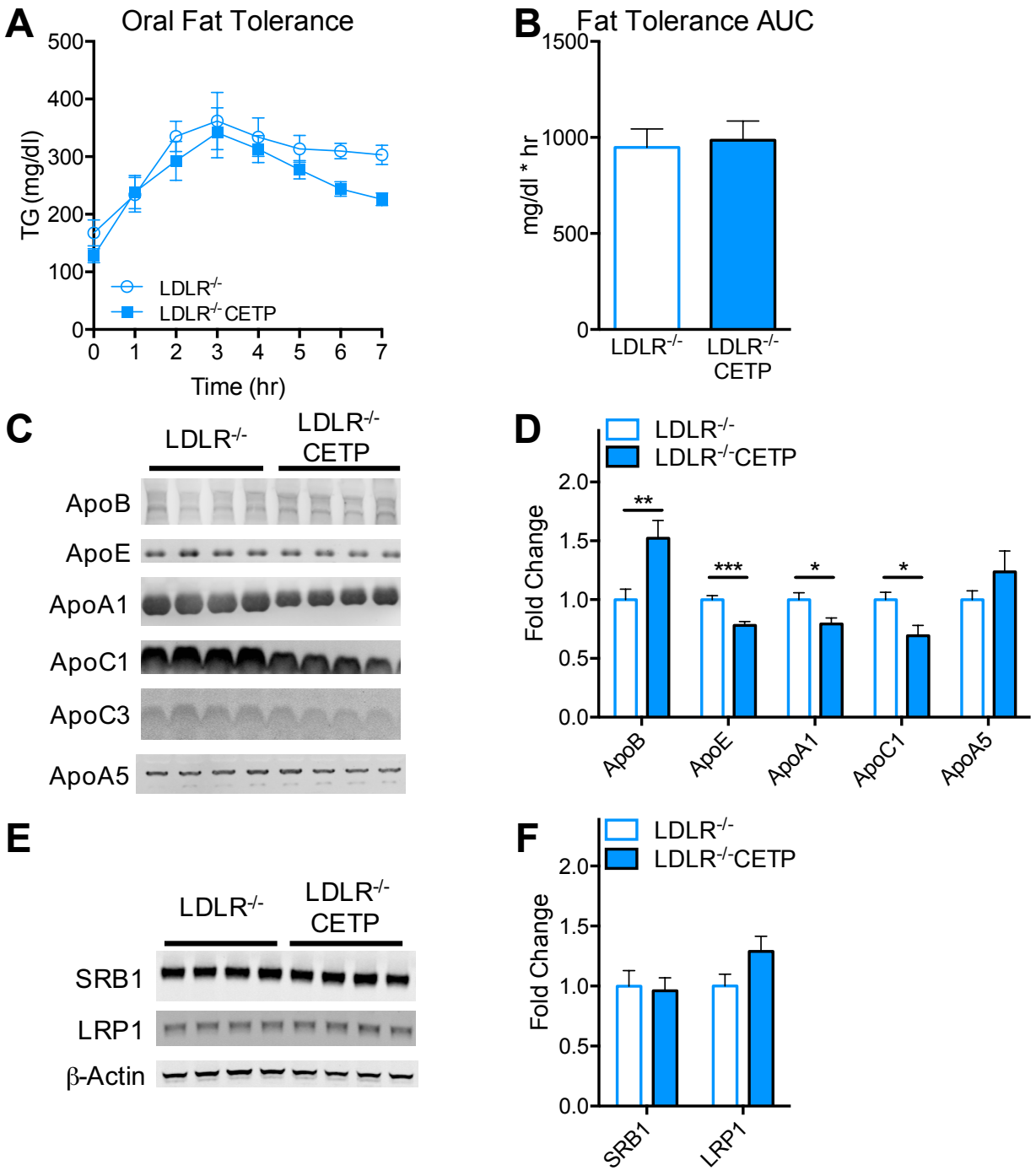

Figure 6.5. LDLR is required for CETP to impair TG clearance in males.
A. Plasma TGs following oral TG bolus (200 μl/mouse) in 12hr fasted LDLR$^{-/-}$ and LDLR$^{-/-}$CETP males. **B.** Area under the curve (AUC) analysis of oral fat tolerance test. **C-D.** Western blotting (C) and quantification (D) of apolipoproteins (*P<0.05, **P<0.01, ***P<0.001, ANOVA n=6-9/group). Apolipoprotein expression was normalized to the average band intensity of LDLR$^{-/-}$ males. **E-F.** Western blotting (E) and quantification (F) of liver lipoprotein uptake receptors (n=6-9/group). Expression of liver proteins were normalized to β-actin. All data are summarized using mean ± SEM.

Low Density Lipoprotein Receptor is Required for CETP to Reduce Liver Cholesterol Content in Males.

In addition to the ability of CETP to regulate liver lipid levels in females, CETP expression resulted in changes in lipid levels in males. CETP expression modestly reduced liver cholesterol content without altering liver TG content. This modest effect of CETP on liver cholesterol content correlated with reduced LDLR expression and required male gonadal hormones, suggesting that male sex-hormone signaling was required for CETP to alter liver LDLR expression and liver cholesterol content. Additionally, since we show that LDLR is a major determinant of the ability of CETP to alter lipid metabolism in both males and females, we lastly wanted to understand whether LDLR was required for CETP to alter liver lipid content in males. Deletion of LDLR did not dramatically raise liver TG content in males, which contrasts with the effect of LDLR on liver TG content in females (Figure 6.6A, compared to Figure 5.4B and Figure 6.3B). CETP did not alter liver TG content in $LDLR^{-/-}$CETP males relative to $LDLR^{-/-}$ males (Figure 6.6A). Deletion of LDLR increased liver cholesterol content by ~50% relative to mice with LDLR (1.70 vs 1.12 μg/mg liver, $LDLR^{-/-}$ males vs. WT males, Figure 6.6B, Figure 5.4A). In the absence of LDLR, CETP failed to reduce liver cholesterol content (Figure 6.6B). Thus, LDLR is required for CETP to reduce liver cholesterol content. Taken together, these results indicate that LDLR is a major regulator of the ability of CETP to alter plasma and liver TG metabolism in females as well as a major regulator of the ability of CETP to alter plasma TG metabolism and liver cholesterol metabolism in males.

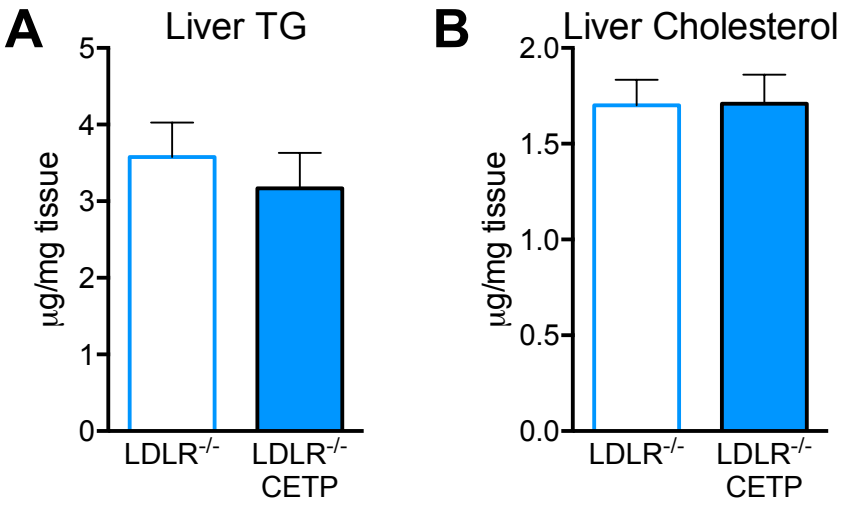

Figure 6.6. LDLR is required for CETP to reduce liver cholesterol content.
A-B. Liver TG content (A) and cholesterol content (B) in $LDLR^{-/-}$ and $LDLR^{-/-}$CETP males (n=6-9/group). All data are summarized using mean ± SEM.

Discussion

The aim of this study was to understand how CETP, a secreted plasma protein, could alter liver TG metabolism. We hypothesized that expression of CETP in mice might allow a novel route of lipid entry to the liver, which might result in changes in hepatic TG metabolism. In line with this thinking, we reasoned that a cell surface receptor regulating TG uptake into the liver may play an important role in CETP-mediated changes in lipid delivery to the liver. In an effort to understand the molecular mechanisms by which CETP alters TG metabolism, we found that LDLR is a major determinant of the ability of CETP to alter TG metabolism in both males and females. In females, LDLR was required for CETP to raise plasma TGs, raise VLDL production, and increase expression and activity of genes involved in VLDL synthesis and assembly in response to estrogen. Additionally, LDLR was partly required for CETP to reduce liver TG content. Unexpectedly, deletion of LDLR reduced liver mRNA levels of β-oxidation genes in females. Thus, LDLR is required for CETP to enhance liver β-oxidation, but CETP may also reduce liver TG content by other mechanisms in the absence of LDLR. In males, LDLR was required for CETP expression to raise plasma TG levels and impair postprandial TG clearance. CETP expression was still able to alter plasma apolipoprotein concentration in males, but these changes did not supersede LDLR in regulation of TG clearance. Thus, LDLR is a major determinant of CETP function on TG metabolism in both males and females.

The requirement of LDLR for CETP to alter TG metabolism is perhaps more straightforward in males than females. In males, CETP expression impaired TG clearance and reduced LDLR expression in liver. Since LDLR promotes TG clearance (137, 138, 323-325, 333), the reduced LDLR expression in CETP males potentially explains why CETP males had impaired TG clearance. Data presented here supports that model (Figure 6.7A). In females, CETP expression resulted in increased VLDL production in response to estrogen, without effects on LDLR expression (Chapters 3 and 4). Previously, LDLR has been shown to decrease VLDL

production (323, 325, 327, 329-332). Our data suggests that CETP requires LDLR to increase VLDL production in response to estrogen in females (Figure 6.7B). Since LDLR was required for CETP to raise VLDL production in response to estrogen, the mechanism of this process may involve disinhibition of LDLR signaling to pathways that govern VLDL production. Further work delineating the signaling pathway connecting LDLR to VLDL production will be necessary to confirm whether CETP alters this signaling pathway in response to estrogen in females.

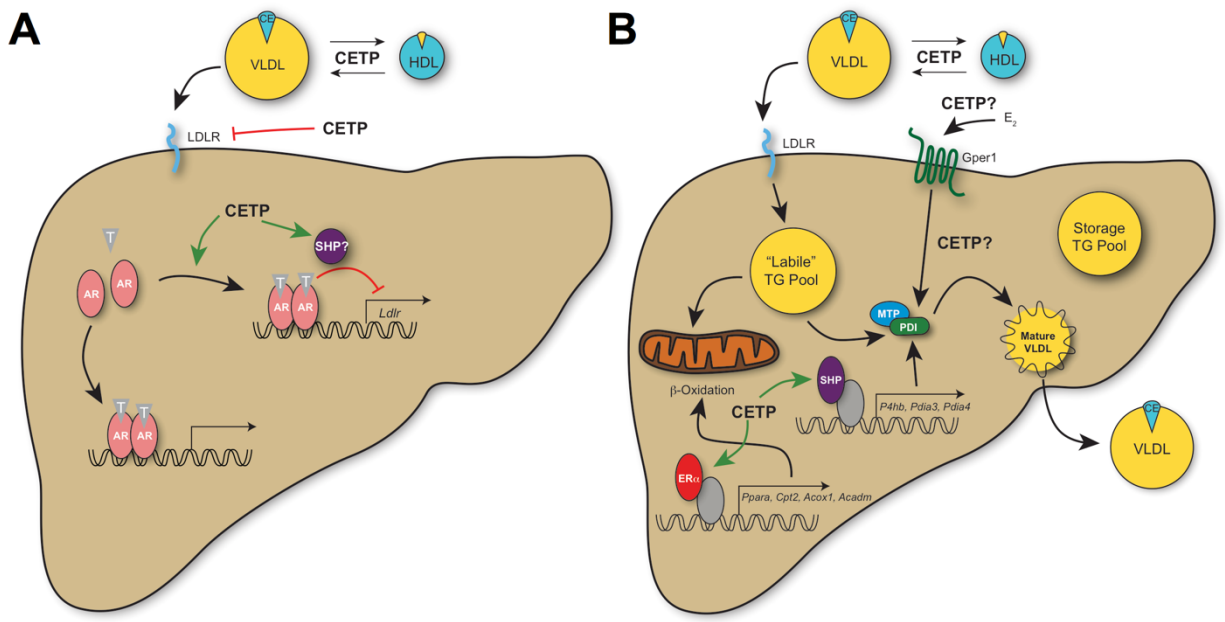

Figure 6.7. Model of how CETP alters TG metabolism in males and females.
A. Model of CETP function on TG metabolism in males. LDLR is required for CETP to impair TG clearance in males. CETP impairs TG clearance through reduced expression of LDLR (Chapter 5). This reduction in LDLR expression requires gonadal hormones. Gonadal hormones do not regulate TG levels in WT mice, indicating that CETP likely alters AR function. One way CETP might utilize gonadal hormones to impair LDLR expression is to increase AR occupancy of the *Ldlr* promoter. CETP-mediated increases in AR promoter occupancy may result in repression of *Ldlr* transcription, resulting in reduced LDLR protein. **B.** Model of CETP function on TG metabolism in females. LDLR is required for CETP to increase VLDL export in response to estrogen. CETP may facilitate lipid uptake by the liver to a "labile" TG pool downstream of LDLR. This labile lipid pool is more prone to incorporation into VLDL particles or more prone to catabolism through β-oxidation. Estrogen treatment promotes TG export as VLDL in CETP females in a process requiring Gper1, SHP and PDI (Chapters 3 and 4). In the absence of LDLR, less TG is mobilized into this "labile" TG pool, and thus the effect of estrogen on VLDL production in CETP is diminished. Furthermore, in the absence of LDLR, less "labile" TG is available for β-oxidation. Thus, any effects of CETP on β-oxidation gene expression are reduced. CETP still reduced liver TG content in the absence of LDLR, but the effect of CETP on β-oxidation gene expression was completely attenuated. Thus, the effect of CETP on liver TG content in the absence of LDLR was likely via a different mechanism than enhanced β-oxidation. CETP may interfere with signaling downstream of LDLR, but these mechanisms are currently unknown.

The requirement of LDLR for CETP to alter liver TG content in females highlights the significance of LDLR in regulating liver TG content in females. Deletion of LDLR has not been shown to regulate liver TG content in males (79, 334). In male mice used in our studies, LDLR did not have a major impact on liver TG content in males (Figure 6.6A, compared to Figure 5.3B). In females, however, deletion of LDLR results in a dramatic increase in liver TG content, especially in the absence of estrogen (Figure 6.3B, compared to Figure 3.6A). Estrogen treatment reduced liver TG content in both $LDLR^{-/-}$ and $LDLR^{-/-}$CETP females, but not to normal levels (Figure 6.3B, compared to Figure 3.6A). This suggests that lipid uptake by LDLR in females results in a generation of or trafficking to a specific pool of TG that is especially susceptible to utilization by the liver, either for β-oxidation or VLDL export (Figure 6.7B). We previously demonstrated the importance of ERα in both estrogen-mediated reductions in liver steatosis and CETP-mediated reductions in liver steatosis (Chapter 4). ERα may govern expression of target genes (i.e. β-oxidation genes) that utilize TG from this LDLR derived pool. Expression of CETP may enhance ERα-mediated expression of β-oxidation targets that access this “labile” pool. Furthermore, with estrogen treatment in CETP females, this labile pool may represent a novel substrate TG droplet that is able to be packaged into VLDL. LDLR or LDLR signaling is required for CETP to move substrate TG into VLDL in response to estrogen, as indicated by the failure to increase PDI activity and expression of genes governing VLDL synthesis in the absence of LDLR.

Although we demonstrate the requirement of LDLR in mediating the effects of CETP on liver and plasma TG in both males and females, CETP still altered expression of certain lipid metabolic targets in both male and female mice. This indicates that some aspects of CETP function are upstream of LDLR. In males lacking LDLR, CETP expression still resulted in changes in plasma apolipoprotein levels. In females lacking LDLR, CETP expression altered the

estrogen response of genes involved in VLDL synthesis and assembly and in genes governing β-oxidation. This indicated that CETP can alter gene expression despite the absence of LDLR. Furthermore, in females lacking LDLR, CETP expression still reduced liver TG content by 25%. This reduction in liver TG content was likely through a different mechanism than enhancing β-oxidation since CETP expression did not increase liver mRNA levels of β-oxidation target genes as it did in the presence of LDLR (Chapter 3). Thus, CETP expression still altered liver TG content in females in the absence of LDLR, but likely *via* a minor pathway since most of the effect of CETP on liver steatosis was abrogated by the deletion of LDLR. Thus, although the deletion of LDLR did not alter the ability of CETP to change lipid metabolic gene expression, deletion of LDLR abrogated the ability of CETP to alter the metabolic fate of TG. This suggests a model whereby CETP expression results in gene expression changes. These gene expression changes then act on TG pools derived from LDLR uptake of lipoproteins (Figure 6.7B). Thus, CETP-mediated gene expression changes in females are less impactful on TG homeostasis in the absence of LDLR and LDLR-derived TG pools. Since CETP seems to directly reduce LDLR expression in males to impair TG clearance, deletion of LDLR prevents CETP-mediated reductions in TG clearance relative to control males because all TG would be cleared through pathways with receptors expressed at similar levels.

The data presented here suggest a broader role for LDLR in regulating VLDL production than previously appreciated. LDLR has been shown to reduce VLDL production as indicated by higher rates of VLDL production in mouse models with genetic deletion of LDLR (327, 329) and in humans with familial hypercholesterolemia (323, 325, 330-332), which is caused by genetic disruption of LDLR function. The presumed mechanism of LDLR reducing VLDL production has been the ability of LDLR to immediately bind and re-uptake newly synthesized VLDL particles. According to this model, lack of LDLR would result in less association of newly synthesized VLDL particles with cell-surface LDL receptors, and thus, more newly synthesized VLDL would

reach circulation. Data presented here, suggest that LDLR is required for CETP to increase VLDL production in response to estrogen. Whether estrogen treatment in CETP mice results in reduced LDLR-mediated reuptake of newly synthesized VLDL or disrupted signaling downstream of LDLR remains to be determined. Several pieces of data, however, suggest that estrogen disrupts signaling downstream of LDLR to alter VLDL production in CETP females. Firstly, in the absence of LDLR, estrogen fails to increase PDI activity in LDLR$^{-/-}$CETP females. If estrogen increased VLDL production by limiting VLDL reuptake by LDLR in CETP females, deletion of LDLR would not be expected to interfere with PDI activity. Deletion of LDLR did, however, prevent estrogen treatment from increasing PDI activity in CETP females. Secondly, deletion of LDLR prevents estrogen-mediated increases in mRNA levels of genes involved in VLDL synthesis and assembly in CETP females. If LDLR did not alter cell signaling, deletion of LDLR would not interfere with estrogen-mediated changes in gene expression in CETP females. We cannot, however, rule out an indirect effect of increased liver cholesterol content in LDLR$^{-/-}$ females as a potential mechanism that interferes with estrogen-mediated gene expression changes in CETP females. Thus, more work understanding LDLR-mediated regulation of VLDL production is needed. In general, these results suggest that expression of CETP alters lipid flux to the liver in a process involving LDLR, which results in gene expression changes that ultimately regulate liver TG metabolism.

Overall, these results indicate that LDLR is a major determinant the effect of CETP expression on TG homeostasis. Additional understanding of how CETP intersects with LDLR function, especially in the context of estrogen, may generate novel targets that alter risk of cardiovascular disease. Although reductions in LDLR abrogate the ability of CETP to raise VLDL production in response to estrogen, more work will be needed to understand if increasing LDLR expression will enhance the ability of CETP to raise VLDL production. This is especially important given that statin therapy, the mainstay of modern cardiovascular disease treatment and prevention, increases liver LDLR expression (319, 335). This is also especially important for

clinical trials of CETP inhibitors, since each clinical trial is designed to test whether CETP inhibition improves risk of cardiovascular disease on top of statin therapy (243, 244). Given that CETP inhibits LDLR expression in impair TG clearance in males, CETP inhibition in the setting of statin therapy would be predicted to be beneficial since both CETP inhibition and statin therapy would act to increase LDLR expression in males. In females, CETP inhibition on top of statin therapy would have a doubly beneficial effect on reducing VLDL production in women since statins themselves reduce VLDL production and since our data suggest that CETP inhibition might also reduce VLDL production in females. CETP inhibitors have been shown to actually increase VLDL production (245, 247), but these studies were conducted mainly in men and these effects were negated by concurrent statin treatment. Since CETP reduces liver TG content in females, the effect of CETP inhibition on liver steatosis in women remains to be determined, especially in the setting of increased LDLR expression induced by statins. Thus, CETP inhibition may be more favorable in men than women if CETP inhibition raises liver TG content in women. A better understanding of how CETP function intersects with LDLR signaling may lead to the identification of novel targets that alleviate risk of cardiovascular disease. It is especially intriguing that a particularly “labile” TG pool may exist that is more prone to β-oxidation or VLDL export. Development of therapies that inhibit movement of this TG pool into VLDL may simultaneously reduce VLDL production and reduce liver TG steatosis, and ultimately reduce risk of cardiovascular disease.

CHAPTER VII

7. CONCLUSIONS AND FUTURE DIRECTIONS

Overview

Cardiovascular disease has been the number one killer of men and women in the United States for nearly 100 years. Great strides have been made in treating and preventing cardiovascular disease with the development of drugs that modify risk factors for cardiovascular disease. Despite this progress, there is still a great deal of “residual” risk of cardiovascular disease, even with currently available treatments (1). Thus, additional work is needed to understand the molecular pathways responsible for this remaining residual risk so that novel pharmacologic agents that can be developed. Since women have lower risk of cardiovascular disease than men (3-7, 9), understanding sex differences may lead to discovery of novel pathways that alleviate risk of cardiovascular disease. Drugs aimed at augmenting pathways that protect women from cardiovascular disease will reduce risk of cardiovascular disease, not only in women, but also in men. Currently, however, the molecular pathways responsible for sex differences in risk of cardiovascular disease remain poorly understood.

Our work has identified the lipid transfer protein, Cholesteryl Ester Transfer Protein (CETP), as a potential mediator of female-specific protection from cardiometabolic disease with obesity (254, 264). My thesis work has established a number of female- and estrogen-specific effects of CETP on triglyceride (TG) metabolism. Although mice naturally lack CETP, we utilized a mouse model that expresses a CETP transgene to determine that CETP is required for estrogen to raise plasma TGs in mice (Chapter 3) (264). Using a number of genetically modified mouse models, I determined that CETP signals through two distinct liver signaling networks to govern

TG metabolism – one involving liver ERα to enhance β-oxidation and reduce liver TG content, and another involving Gper1 and SHP to raise VLDL-TG production in response to estrogen (Chapter 4) (264). I also found that CETP expression in males increased plasma TGs by impairing postprandial TG clearance *via* a mechanism requiring gonadal hormones (Chapter 5). Lastly, I determined that LDLR was a major upstream determinant of the effects of CETP on TG metabolism in both males and females (Chapter 6). In conclusion, my thesis work in the Stafford laboratory identified that CETP utilizes sex hormone signaling to alter liver and plasma TG metabolism *via* a number of novel signaling pathways. Future work aimed at understanding these novel signaling pathways may lead to the discovery of novel therapeutic agents that may alleviate risk of cardiovascular disease.

Chapter 3

Summary of Findings

In Chapter 3, I demonstrated that CETP interacts with estrogen signaling to alter several aspects of metabolism. As a follow up to our previous results demonstrating that CETP expression resulted in female-specific protection from insulin resistance, I demonstrated CETP was required for estrogen to promote glucose tolerance *in-vivo*. Estrogen treatment in women is known to increase plasma TGs by increasing VLDL production, but mechanisms for the hypertriglyceridemic effect of estrogen remain unknown. I demonstrated that CETP is required to recapitulate the hypertiglyceridemic effect of estrogen in mice. In addition to the effect of CETP on VLDL production with estrogen treatment, I also demonstrated that CETP expression results in impaired TG clearance. This CETP-mediated impairment of TG clearance was independent of estrogen treatment. I also demonstrated that expression of CETP results in differential responses to estrogen, leading to estrogen-mediated increases in mRNA expression and activity of genes involved in VLDL synthesis and assembly, especially Protein Disulfide

Isomerase (PDI). In addition to defining the role of CETP in promoting plasma VLDL production in response to estrogen, I also discovered that CETP expression reduced liver TG content in females. This reduction in liver TG content corresponded with increases in measures of β-oxidation. Thus, CETP was required for the hypertriglyceridemic effects of estrogen. Additionally, CETP seems to disrupt liver estrogen signaling, leading to changes in several aspects of TG metabolism in females. This work has established a novel role for CETP in the regulation of TG metabolism and estrogen signaling. My studies in this chapter open several new avenues of research to further investigate the novel signaling pathways regulated by CETP.

Future Directions

(3.1) The role of PDI in estrogen-mediated increases in VLDL in CETP females. PDI is a subunit of MTP and facilitates apoB maturation in the endoplasmic reticulum. Overexpression of PDI is sufficient to facilitate TG secretion, even when MTP levels are low (132, 336), suggesting that PDI may be able to facilitate VLDL production independent of MTP. In CETP females, MTP levels were low compared to WT females, partly explaining why CETP females have lower levels of VLDL production. In response to estrogen, liver PDI activity increased dramatically only in CETP females, with a corresponding increase in VLDL production. Liver PDI activity has provided a reliable metric for the effects of estrogen on VLDL production in CETP females (Chapter 3, 4, 6). Whether this increase in PDI activity is required for estrogen to increase VLDL production, however, remains to be determined. The increase in PDI activity in response to estrogen in CETP females may be incidental to some other effect of estrogen in CETP females. The use of genetic knockouts to study the role of PDI in estrogen treated CETP females is unlikely to be successful both because of the prominent role PDI has in protein homeostasis (337), and also because at least 10 genes are known to have PDI activity (338). Thus, even if a viable knockout model of PDI could be developed in mice, compensation by other genes will

likely confound the development of mouse model lacking PDI activity. The use of inhibitors of PDI activity is an alternative to the development of PDI knockout models. Several inhibitors of PDI activity have been developed (bacitracin, juniferdin, quercetin-3-rutinoside). Quercetin-3-rutinoside is the most selective PDI inhibitor and has been shown to inhibit PDI activity in mouse models (339). The role of PDI activity in mediating the effect of estrogen on VLDL production could be studied in CETP mice using quercetin-3-rutinoside. Ovariectomized CETP females could be given estrogen and then given increasing doses of quercetin-3-rutinoside or vehicle. If PDI is required for estrogen to increase plasma TGs and VLDL production in CETP females, increasing doses of PDI inhibitor should result in dose-dependent reductions in plasma TGs and reductions in VLDL production in CETP females treated with estrogen.

(3.2) The mechanism by which CETP alters liver estrogen regulation of gene expression. A principal finding of my thesis work has been that CETP alters the mRNA response to estrogen. At certain targets, CETP blunts the estrogen response seen in WT females. At other targets, CETP creates a gain-of-function response to estrogen, resulting in either increased or decreased mRNA expression of the target gene. CETP seems to particularly alter the mRNA expression and estrogen response to pathways regulating liver TG metabolism, especially β-oxidation and VLDL production. This suggests that CETP likely alters estrogen receptor function. This work will open a new field of study aimed at how CETP alters sex hormone signaling. In particular, how CETP alters estrogen regulation of gene transcription remains to be determined. To determine whether CETP alters global transcriptional changes in response to estrogen, mRNA-seq can be used to determine global changes in the transcriptome in response to estrogen in WT and CETP females. Additionally, chromatin immunoprecipitation (ChIP)-sequencing (ChIP-Seq) can be used to determine changes to the genome binding locations of RNA Polymerase 2 (Pol2) and ERα. Once the impact of CETP on the genomic

binding locations of Pol2 and ERα is established, motif analysis and targeted co-immunoprecipitation can be used to determine whether certain coactivators/corepressors are more associated with ERα in the presence of CETP. Another way to confirm whether CETP alters ERα binding to classic Estrogen Response Elements (ERE) in the genome would be to compare the luciferase response to estrogen in WT and CETP hepatocytes transfected with a luciferase construct with classical ERE sequences in the promoter or in mice with an ERE-luciferase transgene (340). Once the differential effects of CETP on global and classical ERE sequences in response to estrogen is determined, understanding the mechanisms by which CETP disrupts estrogen receptor signaling may lead to discovery of novel pathways that contribute to the effect of CETP on TG metabolism. CETP could disrupt estrogen regulation of gene expression by at least three mechanisms:

(1) CETP may alter the available nuclear receptor milieu to modify estrogen receptor function. This may result in differential recruitment of estrogen receptors away from “normal” genomic locations and to novel genomic locations. To determine whether CETP alters the available nuclear receptor milieu, nuclear isolates from livers of CETP and WT females can be isolated and subject to targeted immunoblot analysis to measure the nuclear concentration of known ERα coactivators and corepressors. The differential availability of nuclear receptors can also be measured by untargeted proteomic approaches. The differential association of estrogen receptors with cofactors can be determined using targeted immunoblotting or untargeted proteomics from immunoprecipitated estrogen receptors.

(2) CETP may alter intracellular signaling, leading to changes in post-translational modifications of estrogen receptors. Altered post-translational modification of estrogen receptors may lead to conformational changes that alter the binding to genome locations or binding to coactivator/corepressors (341, 342). This can be studied by first understanding whether CETP alters post-translational modifications of estrogen receptors. Estrogen receptors can be

immunoprecipitated from estrogen or vehicle treated WT and CETP females. These immunoprecipitates can then be subject to proteomic analysis relative to an unmodified estrogen receptor. Peptide fragments with increased size will reveal the locations of post-translational modifications to estrogen receptors. If CETP does alter the post-translational modifications of estrogen receptors either at baseline (vehicle treated), or with estrogen treatment, site-directed mutagenesis can be utilized to determine whether these post-translational modifications are required for CETP-mediated changes in estrogen-receptor function.

(3) CETP may alter the promoter availability of target genes by altering chromatin condensation at various genomic locations. Chromatin condensation can regulate DNA accessibility and determine whether a given transcription factor can bind to a given genomic location (343). Whether CETP alters chromatin condensation can be studied by determining the accessible genome regions using DNase-seq in estrogen or vehicle treated WT and CETP females. DNase-seq reveals open chromatin regions, which are available for transcription. If CETP alters chromatin condensation of certain genomic regions, it may explain why estrogen fails to regulate certain target genes and causes novel estrogen regulation of other target genes.

(3.3) The role of CETP in impairing TG clearance in females. In Chapter 3, I determined that expression of CETP impaired postprandial TG clearance in females, and TG clearance did not change with estrogen treatment. Development of therapies that enhance TG clearance may reduce plasma levels of proatherogenic chylomicron- and VLDL-remnant particles in females. TG clearance is regulated by a number of secreted proteins and tissue-specific TG uptake receptors regulate plasma TG clearance (135-143). Whether CETP impairs TG uptake in all tissues remains to be determined. This can be studied by repeating fat tolerance testing described in Chapter 3, 4 and 6 in the presence of a radio-isotope labeled fatty acid tracer and

examining the tracer uptake into tissues. If CETP expression impairs fatty acid tracer uptake in all tissues equally, it may imply that CETP alters the expression of a secreted protein that regulates activity of TG uptake receptors. Global impairments of fatty acid tracer uptake may also suggest that CETP reduces expression of TG uptake receptors in all or most tissues. Alternatively, if CETP expression impairs fatty acid tracer uptake into specific tissues, it would suggest that CETP has tissue-specific effects on TG uptake receptor expression or activity in that tissue. Once the impact of CETP on tissue-specific TG uptake is established, gene expression analysis of secreted proteins and TG uptake receptors can be measured to determine which specific factors are altered by CETP.

Understanding upstream mediators responsible for CETP-mediated impairments in TG uptake may yield novel therapeutic targets to alleviate the deleterious effects of CETP on TG clearance. I demonstrated in Chapter 4 that liver SHP is required for CETP to regulate VLDL production in females. In addition to the known role of SHP in regulating VLDL production, some of my unpublished work in the Stafford lab suggests that liver SHP may also be involved in regulating TG clearance. To determine whether liver SHP is required for CETP to impair TG clearance in females, LKO-SHP and LKO-SHP CETP females can be subjected to oral fat tolerance testing as described in Chapters 3, 5 and 6. Use of a radioisotope labeled fatty acid tracer would help determine if liver SHP is responsible for global or tissue specific deficits in TG uptake as described above.

(3.4) The role of CETP in promoting liver β-oxidation to reduce liver TG content.

Expression of CETP in females dramatically reduces liver TG content through increased liver β-oxidation (Chapter 3) (264). Understanding how CETP promotes liver β-oxidation may lead to the discovery of therapeutic targets that ameliorate hepatic steatosis. Understanding fatty acid substrate preference in CETP versus WT hepatocytes may yield insight into the mechanisms by

which CETP alters β-oxidation since different enzymes are responsible for the metabolism of different substrates. Additionally, different cellular compartments (peroxisomes versus mitochondria) metabolize fatty acids of different sizes. The impact of CETP on fatty acid substrate preference can be measured using radiolabeled short-, medium-, long- and very-long-chain fatty acid tracers. These tracers can be administered to WT and CETP hepatocytes treated with vehicle or estrogen prior to analysis. Oxygen consumed during metabolism of each fatty acid substrate serves as a proxy for fatty acid oxidation rates. If CETP alters fatty acid substrate preference, oxygen consumption should be higher in CETP hepatocytes relative to WT hepatocytes with either short-, medium-, long- or very-long chain fatty acids. CETP may increase oxidation of all of these fatty acid substrates, which would suggest a more general mechanism by which CETP enhances fatty acid oxidation.

In addition to understanding the role of CETP on fatty acid substrate preference, understanding the molecular determinants required for CETP to alter liver β-oxidation will also yield insight into the molecular mechanisms whereby CETP enhances liver β-oxidation. We previously demonstrated that liver ERα was required for CETP to enhance liver mRNA levels of genes involved in β-oxidation. In particular, CETP increased expression of PPARα, which regulates expression of a number of β-oxidation target genes (344, 345). To determine whether PPARα is required for CETP to enhance liver β-oxidation, CETP can be crossed onto a liver specific PPARα knockout mouse (346). Liver TG content and mRNA levels of β-oxidation target genes can be measured to determine the impact of CETP on these targets in the absence of PPARα. Additionally, overexpression of PPARα in the absence of liver ERα could help determine if PPARα signaling is sufficient to mediate the effect of CETP on liver TG content and β-oxidation. This can be done using adenoassociated-virus overexpression of PPARα in LKO-ERα and LKO-ERα CETP females. PPARα expression can be limited to hepatocytes with the use of a liver-specific promoter in the adeno-associated virus vector construct.

Lastly, understanding the role of CETP in intracellular organelle transport to cellular compartments with fatty acid oxidative capacity may also yield insight into the mechanism whereby CETP enhances fatty acid oxidation. Previous *in-vitro* work suggests that CETP can facilitate transfer of lipid to lipid storage droplets (251). It is plausible that CETP may facilitate transfer of lipid to oxidative cellular compartments, like mitochondria or peroxisomes, to enhance β-oxidation in the liver. To understand this process, a novel lipid transfer assay will need to be developed. First generation of a labeled lipid droplet pool would need to be developed using either fluorescent-labeled or radioisotope labeled lipids. Next, isolation of unlabeled peroxisomes and mitochondria would be needed for use as an acceptor of lipid transport. Lastly, addition of cytosol from WT or CETP hepatocytes would be needed. Combination of the labeled lipid droplet "donor", hepatocyte cytosol with and without CETP, and the "acceptor" organelle (either mitochondria or peroxisome) will form the basis of the lipid transfer assay. After incubation of these three components, fatty acid oxidation and lipid transfer can be measured. Fatty acid transfer can be determined based on the tracer accumulation into the target organelle. The target organelle can be separated from other assay components using ultracentrifugation. If a tritiated fatty acid tracer is used to label the donor lipid droplet, fatty acid oxidation can also be approximated from this by measuring the accumulation of tritiated water in the supernatant.

(3.5) The impact of CETP and estrogen on total body substrate preference. Expression of CETP resulted in a dramatic increase in plasma ketone levels with fasting (Chapter 3). This may indicate a preference for fatty acid oxidation as substrate for energy utilization, or this may indicate that CETP expression depletes glucose storage more quickly than wild type controls. It is attractive to speculate that CETP may deplete glucose stores more quickly than WT controls since CETP protects against diet-induced insulin resistance (254) and since CETP expression increases ketone levels with fasting (Chapter 3). These studies were, however, conducted

under different dietary conditions and in mice with and without ovariectomy. Furthermore, CETP increased ketone levels in ovariectomy, a condition where CETP did not alter glucose tolerance. Further understanding of how CETP alters total body substrate preference may yield additional understanding into the global impacts of CETP on total body metabolism. This can be measured *in-vivo* using indirect calorimetry to measure oxygen consumption, carbon dioxide production, energy expenditure, and activity in WT and CETP mice treated with and without estrogen. Since CETP increased plasma ketone levels with fasting, indirect calorimetry can also be used to measure the impact of CETP on fasting. An increase in respiratory exchange ratio would indicate a greater substrate preference for glucose, whereas a decrease in respiratory exchange ratio would indicate a greater substrate preference for fat. Since we show that liver ERα was required for CETP to enhance plasma ketones (Chapter 4), these studies could also be done concurrently in LKO-ERα and LKO-ERα CETP females to determine whether liver ERα was required for CETP to mediate changes in total body substrate preference.

(3.6) The role of CETP in TG function in other tissues. Work in my thesis has largely focused on the role of CETP in altering TG metabolism in the liver. The liver plays an essential role in regulating plasma TG levels since liver TG serves as the source of TG for VLDL. The liver also acts as a major regulator of total body TG uptake. Other tissues however, may also play an important role in the ability of CETP to alter TG metabolism. Previously, transgenic overexpression of CETP only in adipocytes revealed that CETP alters adipose tissue function, resulting in increased plasma apoB, decreased adipocyte size, and decreased adipose content of triglyceride and cholesterol (252). Plasma triglycerides were not different in this study. Thus, CETP may have tissue specific effects on TG metabolism. Several approaches may be used to understand the role of CETP in other tissues. Development of a number of CETP transgenic lines with tissue specific promoters (Albumin-CETP, Ucp1-CETP, Mck-CETP, Villin-CETP)

could be used to study the impact of CETP with expression restricted to certain tissues (liver, brown adipose, muscle, intestine). Alternatively, genetic manipulation of the CETP gene could be used to create an artificial stop sequence that inhibits translation of the CETP gene. If this sequence was manipulated to be flanked by loxP sites, tissue-specific expression of Cre recombinase would result in tissue-specific removal of the stop sequence and generation of tissue-specific CETP expression models. In either case, substantial effort would be necessary to develop novel models of tissue-specific CETP expression. Despite this hurdle, understanding the role of CETP in specific tissues is still warranted to better understand how CETP alters TG metabolism.

In addition to the development of tissue-specific CETP transgenic models, tissue-specific functions of CETP can also be studied from more conventional CETP transgenic models using manipulation of metabolically active tissues *ex-vivo*. Brown adipose tissue is a highly oxidative tissue that can regulate TG clearance (347, 348). Since I have demonstrated that CETP impacts both β-oxidation and TG clearance, CETP may alter brown adipose tissue function to mediate these effects. Furthermore, brown adipose tissue has been shown to contribute to improved glucose metabolism *in-vivo* and in humans (349). Thus, CETP-mediated changes in brown adipose tissue function may mediate a number of effects of CETP on TG and glucose metabolism. It is unclear how CETP-mediated alteration in brown adipose function could simultaneously explain both impaired TG clearance and improved glucose metabolism since increased brown adipose tissue activity is associated with increased TG clearance and improved glucose tolerance. Nonetheless, further study of the impact of CETP on brown adipose tissue is warranted. Understanding whether CETP alters TG uptake into brown adipose tissue using labeled fatty acid tracers during oral fat tolerance testing (described above in (3)) may suggest whether CETP alters brown adipose tissue TG uptake. Additionally, examining how CETP alters expression of TG uptake receptors (LDLR, LPL, etc.), markers of brown

adipose tissue function (*Ucp1*, etc.), and markers of β-oxidation (*Ppara, Pgc1a, Cpt1a, Cpt2,* etc.) will clarify the role of CETP in brown adipose tissue function.

In addition to the potential role of CETP in brown adipose tissue function to promote TG oxidation, CETP may also promote the browning of white adipose tissue, which has been labeled “beiging” of white adipose (350, 351). Understanding whether CETP alters TG uptake in to white adipose tissue depots may indicate whether CETP alters white adipose tissue function. Furthermore, understanding expression of markers of TG uptake and storage (*Lpl, Hsl,* etc.) and beiging (*Prdm16, Cidea, Ppargc1a*, etc.) will help determine whether CETP alters white adipose tissue function or alters beiging of white adipose tissue. Whether beiging of white adipose tissue contributes to TG clearance remains an unanswered question in the literature. I would hypothesize that factors increasing beiging of white adipose tissue likely increase TG clearance. Therefore, CETP may negatively impact beiging of white adipose tissue to impair TG clearance.

(3.7) The selective action of CETP inhibitors on aspects of CETP function. My thesis work has demonstrated several novel functions of CETP on metabolism. While improving glucose metabolism (254), reducing liver steatosis (Chapter 3) and increasing exercise capacity with obesity (352) are potentially beneficial effects of CETP, CETP also causes several negative effects, including impaired TG clearance (Chapters 3 and 5), increased VLDL production with estrogen (Chapters 3 and 4), and reduced HDL cholesterol levels. Thus, CETP actions have a complex impact on risk factors for cardiovascular disease. The multitude of CETP functions suggest a potential reason why CETP inhibitors have failed to improve risk of cardiovascular disease. If CETP inhibitors inhibit both the beneficial and harmful functions of CETP, the net result of CETP inhibition on risk of cardiovascular disease may be neutral. While three CETP inhibitors (torcetrapib, dalcetrapib and evacetrapib) have failed to show a benefit to cardiovascular disease risk (240-242), two CETP inhibitors (anacetrapib, TA-8995) remain in clinical development (243, 244). The success or failure of these CETP inhibitors and future

CETP inhibitors may rest in the selectivity of these agents to inhibit certain aspects of CETP function. An ideal CETP inhibitor should only inhibit the negative effects of CETP function (impaired TG clearance, increased VLDL production with estrogen treatment) and permit the beneficial effects of CETP function (reduced liver steatosis, improved glucose tolerance, increased exercise capacity). The effect of each CETP inhibitor can be measured on these aspects of metabolism by dosing CETP mice with each CETP inhibitor and measuring the effects of these drugs on various effects on metabolism. A major unanswered question is whether CETP inhibitors alter liver TG content. This would likely prohibit the clinical development of any CETP inhibitor. Understanding the impact of CETP inhibitors on liver TG content in CETP mice, especially females, may provide a novel preclinical screening tool that may allow for the development of selective CETP inhibitors.

Chapter 4

Summary of Findings

In Chapter 4, I demonstrated that CETP signals *via* distinct liver nuclear receptor networks to alter liver and plasma TG metabolism in females. In Chapter 3, I demonstrated that estrogen was required for CETP to increase VLDL production in CETP females. Liver TG provides the substrate for VLDL-TG production. In liver, Estrogen Receptor α (ERα) is the most highly expressed estrogen receptor and is involved in regulating a number of lipid metabolic pathways in the liver. Therefore, I determined whether liver ERα was required for CETP to alter liver and plasma TG metabolism. I found that liver ERα was required for CETP expression to increase β-oxidation and reduce liver TG content. Unexpectedly, liver ERα was dispensable for estrogen to raise VLDL-TG production in females, suggesting that estrogen may signal through a less-highly expressed estrogen receptor to increase VLDL production in CETP females. I found that Gper1 signaling was required for estrogen to increase VLDL production in CETP females. Additionally,

I found that the nuclear receptor Small Heterodimer Partner (SHP), a nuclear receptor involved in VLDL production and a number of other metabolic pathways, was required for estrogen to increase VLDL production in CETP females. Thus, CETP alters liver and plasma TG metabolism through at least two distinct liver signaling networks – one involving ERα to enhance β-oxidation and reduce liver TG content, and another involving Gper1 and liver SHP to increase VLDL production in response to estrogen. Further understanding of the molecular signaling changes caused by CETP can be studied in several future experiments discussed below.

Future Directions

(4.1) Determine the role of ERα signaling versus ERα gene transcription in CETP-mediated changes in β-oxidation. Results from Chapter 4 demonstrate that ERα is required for CETP to enhance β-oxidation. ERα can alter cell metabolism both through intracellular signaling and by altering gene transcription (98, 99). ERα can alter cell signaling through an estrogen-responsive ERα isoform localized to the cell membrane. This membrane localized isoform of ERα has been shown to be important in several aspects of metabolism. Unique mouse models with either membrane-only ERα (MOER) and nuclear-only ERα (NOER) have been developed to study ERα function (113, 353). To determine whether ERα mediated cell signaling or ERα mediated changes in gene transcription are required for CETP to enhance β-oxidation, CETP can be crossed onto MOER and NOER. Membrane associated ERα signaling has been shown to mediate the effect of estrogen on liver lipid content. If membrane function of ERα is required for CETP to enhance β-oxidation and lower liver TG content, MOER-CETP female mice will have lower liver TG content compared to control MOER females and NOER-CETP female mice will not have lower liver TG content relative to control NOER females. Conversely, if nuclear ERα is required for CETP to enhance β-oxidation and reduce liver TG

content, MOER-CETP female mice will not have lower liver TG content relative to control MOER females, and NOER-CETP female mice will have lower liver TG content relative to control NOER females. Deciphering whether ERα cell signaling versus ERα-mediated gene expression changes contributes to CETP-mediated reductions in liver TG content will clarify the mechanisms by which CETP can alter TG metabolism.

(4.2) The role of CETP in altering Gper1 signaling to promote VLDL production in response to estrogen. In Chapter 4, I demonstrated that pre-treatment with a Gper1 antagonist completely attenuates the effect of estrogen on VLDL production in CETP females. Whether Gper1 signaling is sufficient to recapitulate the effect of estrogen on VLDL production in CETP females remains to be determined. This can be studied with the use of a commercially available Gper1 agonist (G-1, Cayman Chemical). Studies examining VLDL production in WT and CETP females treated with vehicle or the Gper1 agonist, G-1, will determine whether Gper1 signaling is sufficient to promote VLDL production in CETP females. The role of Gper1 in mediating estrogen's effect on promoting VLDL production in CETP females can be further confirmed by crossing CETP onto a Gper1 knockout mouse (123). If Gper1 is required for CETP to raise VLDL production in response to estrogen, estrogen will fail to increase VLDL production in CETP mice lacking Gper1.

To determine if CETP alters Gper1 signaling, second messenger activity can be measured in hepatocytes in response to Gper1 selective agonists and antagonists. Gper1 has been shown to increase both calcium and cyclic AMP in response to estrogen and Gper1 agonists. If CETP alters Gper1 signaling, CETP should alter intracellular levels of calcium or cyclic AMP in response to Gper1 agonism. Since hepatocytes do not express high levels of Gper1 (118), forced expression of Gper1 and CETP in cells lacking both Gper1 and CETP may be necessary to understand the role of CETP in altering Gper1 signaling. These studies could be done by transfecting CHO or HEK293 cells with Gper1 and CETP. Following transfection of these genes,

intracellular calcium and cyclic AMP can be measured in response to Gper1 agonists and antagonists to determine how CETP alters Gper1 signaling.

Determining whether Gper1 regulates estrogen-mediated increases in SHP expression may confirm a novel signaling pathway induced by estrogen in CETP females. In Chapter 4, I demonstrated that Gper1 signaling and liver SHP were independently required for estrogen to increase VLDL production in CETP females. One previous study has suggested a connection between Gper1 signaling and SHP (293), but this study suggests that Gper1 signaling reduces SHP expression. My work suggests that Gper1 signaling may enhance SHP expression. CETP may therefore impair Gper1 signaling to promote SHP expression. Understanding how Gper1 signaling alters SHP expression can be done using Gper1 agonists and antagonists in WT and CETP mice. Following treatment WT and CETP females with vehicle of Gper1 agonist, liver SHP mRNA can confirm the role of Gper1 signaling in regulating SHP. In my current model, I would expect that Gper1 signaling increases liver SHP mRNA expression. Alternatively, Gper1 signaling may promote VLDL production by enhancing PDI activity independent of changes in SHP expression. Measuring liver SHP mRNA and liver PDI activity would implicate Gper1 in regulating liver SHP mRNA or PDI activity. Furthermore, administration of Gper1 agonists in mice lacking liver SHP can confirm whether Gper1 signaling requires liver SHP to promote VLDL production in CETP females. If SHP was not required for Gper1 signaling to promote VLDL production in CETP mice, administration of a Gper1 agonist should increase VLDL production in LKO-SHP CETP females relative to vehicle treated LKO-SHP CETP females.

(4.3) The role of CETP in altering SHP function. In Chapter 4, I demonstrated that liver SHP is required for CETP to increase VLDL production in response to estrogen. I also demonstrated that expression of CETP results in a modest enhancement of liver SHP mRNA expression in response to estrogen. My previous work shows that CETP enhances the ability of insulin to increase liver SHP mRNA (254). Understanding how CETP regulates SHP function

will further delineate the mechanisms by which CETP alters signaling to promote VLDL production. We previously suggested that CETP promotes bile acid signaling to increase insulin sensitivity in CETP females (254). Estrogen is also known to increase liver bile acid content by reducing bile acid export expression (291). CETP may therefore enhance liver SHP expression by altering bile acid signaling. To understand whether CETP alters bile acid signaling to alter liver SHP function in response to estrogen, CETP transgenic mice can be bred onto mice with liver specific deletion of Farnesoid X Receptor (FXR) or Fibroblast Growth Factor Receptor 4 (Fgfr4). Liver FXR and Fgf4r signaling are major upstream regulators of SHP expression and bile acid signaling (354, 355). Understanding whether CETP can enhance SHP expression in response to estrogen in the absence of either liver FXR or liver Fgfr4 will determine whether bile acid signaling is a mechanism by which CETP alters SHP function. If bile acid signaling is required for CETP to alter SHP function in response to estrogen, deletion of FXR or Fgf4r will attenuate the effects of estrogen on liver SHP expression in response to estrogen in CETP females. Liver TG content and plasma TG metabolism can also confirm the role of bile acid signaling in CETP-mediated changes in TG metabolism. Although CETP may alter SHP function *via* other mechanisms, the bile acids are the most well characterized regulators of SHP. Therefore, understanding whether FXR and Fgfr4 are required for CETP to alter SHP expression in response to estrogen treatment will help decipher how CETP alters SHP function and ultimately how CETP contributes to increased VLDL production in response to estrogen.

Chapter 5

Summary of Findings

In Chapter 5, I demonstrated that CETP alters TG metabolism in males through a pathway dependent on gonadal hormones. In Chapters 3 and 4, I demonstrated that CETP altered liver and plasma TG metabolism in females through several distinct pathways, some of which were

independent of estrogen treatment and independent of estrogen receptor signaling, suggesting that certain effects of CETP may be generalizable to males. I discovered that CETP expression also increased plasma TGs in males, but by a different mechanism than in females. Whereas CETP expression resulted in increased VLDL-TG production in females, I found that CETP expression impaired plasma TG clearance in males. This effect of CETP expression on impairing TG clearance in males corresponded with reduced liver expression of lipoprotein uptake receptors LDLR and SRB1. Additionally, I determined that gonadal hormones were required for CETP to impair postprandial TG clearance and liver LDLR and SRB1 expression in males. This effect of CETP on TG metabolism in males was likely independent of aromatization of testosterone to estrogen since liver ERα was not required for CETP to raise plasma TGs in males. Thus, CETP generates a novel response to gonadal hormones in males, similar to the effect of CETP on estrogen signaling in females. Future work aimed at understanding how CETP alters male gonadal hormone signaling is discussed below.

Future Directions

(5.1) The role of CETP in altering lipoprotein uptake receptor expression. In Chapter 5, I demonstrated that expression of CETP in males results in reduced liver expression of both LDLR and SRB1, two lipoprotein receptors that are major regulators of lipid uptake (137, 138, 323-325, 356). I also demonstrated that gonadal hormones were required for CETP expression to alter the liver expression of these lipoprotein receptors. To determine how CETP alters expression of these lipoprotein uptake receptors, liver mRNA expression of LDLR and SRB1 can be measured from WT and CETP male mice to help determine whether this difference in expression was at the transcriptional or post-transcriptional level. If mRNA for LDLR or SRB1 is lower in CETP mice than WT mice, it would suggest that CETP alters either mRNA transcription or mRNA stability. Since gonadal hormones were required for CETP to reduce LDLR expression, CETP may impact androgen receptor (AR) signaling to reduce liver expression of

LDLR and SRB1. If CETP expression results in reduced mRNA levels of LDLR or SRB1, ChIP-qPCR can be used to determine whether CETP alters Pol2 promoter occupancy of LDLR or SRB1 and whether CETP alters AR occupancy of these targets.

If mRNA expression of LDLR and SRB1 is similar between WT and CETP males, it may suggest that CETP impacts translational or post-translational regulation of LDLR and SRB1 protein. Translational efficiency can be measured using radioisotope-labeled amino acid incorporation into LDLR and SRB1. Following various labeling times, immunoprecipitation of LDLR or SRB1 can be done from liver or hepatocytes with and without CETP. Accumulation of tracer into LDLR or SRB1 can serve as an estimation of translational efficiency of LDLR and SRB1 protein synthesis. Reduced protein synthesis of LDLR or SRB1 may explain how CETP reduces protein levels of these targets. Conversely, CETP may alter the post-translational rate of clearance of these targets. PCSK9 is a major posttranslational regulator of clearance of LDLR. Increased or decreased PCSK9 expression leads to increased or decreased catabolism of LDLR protein in liver, respectively. Liver PCSK9 levels were actually lower in CETP males, which would tend to increase LDLR protein levels. Nonetheless, CETP may reduce liver LDLR expression by other mechanisms. To determine whether CETP increases post-translational catabolism of LDLR or SRB1 a pulse-chase approach can be used to measure the post-translational catabolism of these proteins. If CETP enhances LDLR or SRB1 catabolism, radioactivity in these targets will decline more quickly in CETP males than in WT males. Since gonadal hormones were required for CETP to reduce expression of LDLR and SRB1, non-genomic signaling of AR may contribute to any translational or post-translational effects of CETP on LDLR and SRB1 expression.

(5.2) The role of CETP in TG uptake into extrahepatic tissues. In Chapter 5, I demonstrate that CETP impairs postprandial TG uptake. I focused on liver expression of lipoprotein uptake receptors as a potential mechanism to explain how CETP impairs TG

clearance. It is plausible, however, that CETP alters TG uptake into other tissues. The tissue-specific effects of CETP on TG uptake can be measured using a radio-isotope labeled fatty acid tracer in the setting of an oral fat tolerance test. Tracer uptake into various tissues is proportional to TG uptake by those tissues. Measuring fatty acid tracer uptake into various tissues in WT and CETP males will determine whether CETP impairs tissue-specific TG uptake or whether CETP impairs TG uptake globally. If CETP impairs TG uptake globally, it may suggest that CETP-mediated changes in secreted factors, like ApoE, contribute to CETP-mediated impairment of TG metabolism. In addition to measuring fatty acid tracer uptake into tissues to approximate the effect of CETP on TG uptake by other tissues, LPL activity in muscle and adipose tissue can be measured in WT and CETP males. LPL is a major regulator of lipid uptake in muscle and adipose tissue (140). Therefore, measuring LPL activity in these tissues may yield insights in to potential mechanisms by which CETP impairs TG clearance in males.

(5.3) The role of CETP in male gonadal hormone signaling. In Chapter 5, I determined that gonadal hormones were required for CETP to alter TG uptake. I demonstrated that liver ERα was dispensable for the effect of CETP on raising plasma TGs, suggesting that CETP impaired TG clearance *via* a mechanism dependent on testosterone and not testosterone aromatization to estrogen. To confirm that testosterone is indeed required for this effect, TG clearance can be measured in gonadectomized WT and CETP males treated with vehicle or testosterone with an aromatase inhibitor or use of a non-aromatizable testosterone analog, such as 5α-dihydroxytestosterone.

To determine whether liver AR signaling is required for CETP to impair TG clearance in males, CETP mice can be bred with mice with a liver specific AR deletion (357). Liver AR has been shown to be important regulator of liver steatosis and plasma TG levels (357), and thus, may be an important determinant of CETP-mediated impairment of TG metabolism in males. If

liver AR is required for CETP to impair TG clearance, CETP expression will fail to impair TG clearance in the absence of liver AR compared to control males lacking liver AR. It is plausible, however, that liver AR is not required for CETP to impair TG clearance despite the fact that gonadal hormones were required for CETP to impair TG clearance in males. In females, liver ERα was dispensable for the effect of CETP on regulation of plasma TGs. It may therefore be advantageous to first study whether CETP impairs TG metabolism in a total body AR knockout rather than a liver-specific AR knockout. This would confirm that gonadal hormone action through AR is required for CETP to impair TG clearance. Tissue-specific re-expression of AR using either genetic or viral methods can help delineate in which tissue AR is required for CETP to impair TG clearance.

Since gonadal hormones are required for CETP to impair TG clearance in males, CETP may alter transcriptional responses of AR. It is plausible for CETP to alter AR function *via* a variety mechanisms similar to those discussed above (3.2). To determine whether CETP alters transcription of classic AR target genes, promoter-luciferase constructs with classical Androgen Response Elements (AREs) in the promoter can be transfected into CETP and WT primary hepatocytes. Following treatment with vehicle or testosterone, luciferase activity should approximate classic AR-mediated transcription. If CETP alters AR-mediated transcription to classical ARE sequences, CETP should increase or decrease luciferase activity detected from hepatocytes containing these constructs. CETP may alter AR promoter occupancy, posttranslational modifications of AR or chromatin availability to mediate novel responses to gonadal hormones. To confirm that CETP alters transcriptional responses or chromatin availability, ChIP-seq for Pol2 can confirm whether CETP alters the genome localization of the transcriptional machinery. This can be combined with ChIP-seq for AR to understand whether CETP alters genomic localization of AR. Co-immunoprecipitation followed by targeted immunoblotting can determine whether CETP alters the association of AR with other known coactivators/corepressors, like Steroid Receptor Coactivator-1 (SRC-1), CREB Binding Protein

(CBP) or p300. Untargeted approaches, such as shot-gun proteomics, can be used to identify whether CETP expression creates novel associations of AR to other transcription factors. Further understanding of how CETP alters AR function in males will complement studies aimed at understanding how CETP alters estrogen function in females. Comparing gene lists generated from global approaches aimed at probing the effect of CETP on AR and ERα may yield insight into common mechanisms by which CETP alters sex hormone signaling and TG metabolism in both males and females. A better understanding of how CETP alters gonadal hormone signaling in both males and females may potentially identify novel therapeutic targets for the treatment of cardiovascular disease.

Chapter 6

Summary of Findings

In Chapter 6, I discovered that LDLR is a major determinant of CETP action on TG metabolism in both males and females. Using mice with genetic deletion of LDLR, I determined that LDLR was required for CETP to raise VLDL production in response to estrogen in females. In the absence of LDLR, estrogen failed to increase liver mRNA expression and activity of genes involved in VLDL synthesis and assembly. Additionally, in the absence of LDLR, the effect of CETP on liver TG content was largely attenuated in females. Furthermore, in the absence of LDLR, CETP failed to enhance liver β-oxidation gene expression, indicating that CETP may alter liver TG content by other minor pathways in the absence of LDLR. In the absence of LDLR, CETP still altered how estrogen regulated mRNA expression of genes involved in VLDL synthesis and assembly and β-oxidation. In males, LDLR was required for CETP to raise plasma TGs and impair TG clearance. In the absence of LDLR, CETP expression still altered plasma apolipoprotein concentration. Thus, LDLR is a major upstream determinant of CETP action on TG metabolism. CETP expression, however, was still able to alter expression

of lipid metabolic targets despite the absence of LDLR in both males and females. These results suggest a model whereby CETP alters gene expression and these CETP-mediated gene expression changes act on a specific liver TG substrate pool derived from LDLR to alter liver and plasma TG metabolism. Future work aimed at understanding the intersection of CETP and cell surface TG uptake receptors are described below.

Future Directions

(6.1) The role of extracellular versus intracellular CETP in altering TG metabolism. In Chapter 6, I discovered that LDLR was a major upstream determinant of CETP-mediated changes in TG metabolism. This suggests that uptake of lipid by specific cell-surface receptors governs the effects of CETP on TG metabolism. In addition, this may also suggest that extracellular actions of CETP on modifying lipid content of lipoproteins may dictate how lipoproteins are taken up by tissues. If such a model of CETP function is true, extracellular CETP action on modifying lipid content of lipoproteins may be sufficient for CETP to alter TG metabolism. To determine if extracellular CETP is sufficient to alter TG metabolism, WT mice can be treated with control peptide or CETP protein administered by subcutaneous, osmotic mini-pumps. Increasing doses of CETP can determine the CETP protein concentration at which CETP is sufficient to alter TG metabolism. This can be done in males or ovariectomized females with estrogen or vehicle treatment. Plasma TGs and liver mRNA expression can be compared to previous results to determine whether extracellular CETP can recapitulate the effects of transgenic CETP in mice. If extracellular CETP protein administration does not impact TG metabolism, it may suggest intracellular CETP function dictates the ability of CETP to alter TG metabolism. Alternatively, if extracellular CETP fails to recapitulate the effects of transgenic CETP expression on TG metabolism, it may suggest that transgenic CETP expression may alter some aspect of development that governs TG metabolism.

(6.2) The role of other lipoprotein receptors in CETP-mediated changes in TG metabolism. In Chapter 5, I demonstrated that CETP expression reduces liver protein expression of LDLR and SRB1. In Chapter 6, I demonstrate that LDLR is a major upstream determinant of CETP action on TG metabolism. SRB1 may still play a part in CETP-mediated changes in TG metabolism. SRB1 is important for both lipoprotein uptake, especially HDL, and also for VLDL production (358, 359). Thus, SRB1 represents an important cell surface receptor that regulates uptake and production of lipoproteins, and therefore, may be another determinant of CETP-mediated alteration of TG metabolism. To determine if SRB1 is required for CETP to alter TG metabolism, CETP can be crossed onto mice with global deletion of SRB1. Studies similar to those completed in Chapter 6 can be done in male and female mice to characterize the impact of SRB1 on CETP-mediated changes in TG metabolism.

(6.3) The role of estrogen in modulating LDLR regulation of VLDL production in CETP females. In Chapter 6, I determined that LDLR was required for estrogen to raise VLDL production in CETP females. LDLR has been previously shown to regulate VLDL production in mice lacking LDLR and in humans with genetic deficiency of LDLR (323, 325, 327, 329-333). The presumed mechanism by which LDLR regulates VLDL production is by reuptake of newly synthesized VLDL particles prior to release of VLDL into systemic circulation. This biophysical mechanism does not, however, explain how deletion of LDLR disrupts estrogen regulation of PDI activity or estrogen-regulated changes in liver mRNA in CETP females. For CETP to increase VLDL production in response to estrogen by this mechanism, estrogen treatment in CETP mice must somehow disrupt the interaction of LDLR with newly synthesized VLDL. To determine whether inhibition of LDLR-mediated VLDL reuptake is the mechanism by which estrogen promotes VLDL production in CETP mice, systemic pre-treatment of CETP or WT mice with an LDLR antibody can be used to disrupt LDLR binding (333). Administration of this

LDLR antibody can be given before administration of Triton-WR1339 in estrogen or vehicle treated WT and CETP females as in Chapters 3 and 4.

Conclusion

The work presented here has led to a number of novel discoveries surrounding the role of CETP in TG metabolism. The principal finding of my thesis is that CETP disrupts sex hormone signaling in both males and females to alter liver and plasma TG metabolism. My initial discovery that estrogen requires CETP to raise plasma VLDL production in females led me to discover that CETP disrupts at least two liver signaling networks to alter liver and plasma TG metabolism in females. I also found that CETP required gonadal hormones to impair postprandial TG clearance in males. Lastly, I found that LDLR is a major determinant of the ability of CETP to alter TG metabolism in both males and females. My work has led to the discovery of a variety of novel signaling pathways induced by CETP, highlighting the complexity of the effects CETP has on TG homeostasis and liver sex hormone signaling. Future work aimed at understanding how CETP alters ERα signaling in females and AR signaling in males will lead to a deeper understanding of the role of CETP in sex hormone signaling. Further understanding of novel signaling pathways regulated by CETP may lead to the discovery of novel targets for the treatment of cardiovascular disease.

Understanding the role of CETP in TG metabolism in male and female transgenic mice has advanced the understanding of sex-differences in risk of cardiovascular disease, but important gaps still remain. Certain studies suggest that CETP or genetic variation in *CETP* may contribute to sex-specific functions in humans (257, 259, 305, 306), but the hypothesis that CETP contributes to sex-differences in risk for cardiovascular disease remains to be tested in humans. Since women are protected from cardiovascular disease relative to men, understanding sex-differences may lead to novel agents that reduce risk of cardiovascular

disease. Drugs aimed at augmenting pathways that target the female protection from cardiovascular risk have a tremendous potential to treat cardiovascular disease. Currently, however, no drugs exist to modify this pathway. Additionally, understanding of these pathways remains poorly understood. My work suggests that understanding CETP action on sex hormone signaling may play an important role in sex-differences in risk for cardiovascular disease. Future work aimed at understanding CETP function may lead to discovery of novel pathways contributing to sex differences in risk of cardiovascular disease.

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