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ORIGINAL ARTICLE

Cholesteryl Ester Transfer Protein Impairs Triglyceride Clearance *via* Androgen Receptor in Male Mice

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Abstract Elevated postprandial triacylglycerols (TAG) are an important risk factor for cardiovascular disease. Men have higher plasma TAG and impaired TAG clearance compared to women, which may contribute to sex differences in risk of cardiovascular disease. Understanding mechanisms of sex differences in TAG metabolism may yield novel therapeutic targets to prevent cardiovascular disease. Cholesteryl ester transfer protein (CETP) is a lipid shuttling protein known for its effects on high-density lipoprotein (HDL) cholesterol levels. Although mice lack CETP, we previously demonstrated that transgenic CETP expression in female mice alters TAG metabolism. The impact of CETP on TAG metabolism in males, however, is not well understood. Here, we demonstrate that CETP expression increases plasma TAG in males, especially in very-low density lipoprotein (VLDL), by impairing postprandial plasma TAG clearance compared to wild-type (WT) males. Gonadal hormones were required for CETP to impair TAG clearance, suggesting a role for sex hormones for this effect. Testosterone replacement in the setting of gonadectomy was sufficient to restore the effect of CETP on TAG. Lastly, liver androgen receptor (AR) was required for CETP to increase plasma TAG. Thus, expression of CETP in males raises plasma TAG by impairing TAG clearance *via* testosterone signaling to AR. Further understanding of how CETP and androgen signaling impair TAG clearance may lead to novel approaches to reduce TAG and mitigate risk of cardiovascular disease.

Keywords Androgen receptor · Cholesteryl ester transfer protein (CETP) · Testosterone · Triglyceride (TAG)

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Abbreviations

AAV8	adeno-associated virus serotype 8
ApoA1	apolipoprotein A1
ApoA5	apolipoprotein A5
ApoB	apolipoprotein B
ApoC1	apolipoprotein C-I
ApoC2	apolipoprotein C-II
ApoC3	apolipoprotein C-III
ApoE	apolipoprotein E
AR	androgen receptor
AUC	area under the curve
CETP	cholesteryl ester transfer protein
FPLC	fast-performance liquid chromatography
GDX	gonadectomized
HDL	high-density lipoprotein
LDL	low-density lipoprotein
LPL	lipoprotein lipase
LRP1	LDLR-related protein
shSCR	scrambled shRNA
shRNA	short hairpin RNA

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SRB1 scavenger receptor B-I
TAG triglyceride
VLDL very low-density lipoprotein
WT wild type

Introduction

Elevated plasma triglyderides (TAG) are an important risk factor for cardiovascular disease in both men and women (Bansal et al., 2007; Castelli, 1986; Freiberg et al., 2008; Patel et al., 2004; Sprecher et al., 2000). Men have higher plasma TAG and lower TAG clearance compared to women (Cianflone et al., 2004; Couillard et al., 1999; Horton et al., 2002; Mittendorfer et al., 2003; Schaefer et al., 1994). Both overproduction of TAG and reduced clearance of TAG contribute to increasing TAG levels and increasing risk of cardiovascular disease (Boquist et al., 1999; Ginsberg et al., 1995; Groot et al., 1991; Sharrett et al., 1995; Sposito et al., 2004; Teno et al., 2000). The role of plasma TAG 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 TAG (D'Agostino et al., 2004; Tirosh et al., 2008). Human genetic approaches have confirmed that lifelong changes in TAG contribute to risk of cardiovascular disease (Holmes et al., 2015). Impaired postprandial TAG clearance is associated with risk of cardiovascular disease and is more predictive of cardiovascular disease risk in men than women (Boquist et al., 1999; Carstensen et al., 2004; Ginsberg et al., 1995; Groot et al., 1991; Sharrett et al., 1995).

TAG clearance is regulated by a number of tissue receptors and secreted proteins (Rosenson et al., 2014). Dietary TAG are packaged into large, TAG-rich lipoproteins called chylomicrons, which contain apolipoprotein B (ApoB). Clearance of TAG by holoparticle lipoprotein uptake is controlled by tissues expressing lipoprotein receptors including low-density lipoprotein receptor (LDLR), LDLR-related protein (LRP1) and scavenger receptor B-I (SRB1) (Gordts et al., 2009; Ishibashi et al., 1996; Kypreos and Zannis, 2006; Wang et al., 1998). Reduced expression of these uptake receptors can impair TAG clearance. Additionally, peripheral lipases, both hepatic lipase and lipoprotein lipase (LPL) regulate clearance of TAG from lipoproteins into tissues (Hegele et al., 1993; Wang and Eckel, 2009). Activity of these lipases is modulated by several circulating proteins bound to the surface of plasma lipoproteins, including apolipoprotein C-I (ApoC1), apolipoprotein C-II (ApoC2), apolipoprotein C-III (ApoC3), apolipoprotein E (ApoE), Apolipoprotein A5 (ApoA5), Angiopoietin-like 3, and 4 (Angptl3 and Angptl4) (Rosenson et al., 2014). Thus, secreted proteins and tissue expression of lipoprotein receptors regulate plasma TAG clearance. Metabolic pathways that coordinate expression of these TAG regulatory factors are currently not well understood. Better understanding of pathways regulating these targets could result in novel therapeutic targets that enhance TAG clearance and ameliorate risk of cardiovascular disease.

Cholesteryl ester transfer protein (CETP) is a lipid transfer protein that shuttles TAG and cholesteryl ester between plasma lipoproteins. Humans express CETP, but mice naturally lack CETP expression. Transgenic expression of the CETP transgene in mice results in a more human-like lipoprotein distribution (Pape et al., 1991). Thus, transgenic expression of CETP in mice represents a model of lipid metabolism that more closely resembles human physiology. Additionally, use of transgenic CETP in mice allows for the study of novel functions of CETP without the toxic or off target effects of CETP inhibitors (Barter et al., 2007). Although CETP is known for its plasma effects on lowering plasma high-density lipoprotein (HDL) cholesterol, we have previously shown that transgenic CETP expression in female mice impacts liver glucose and TAG metabolism with in females in an estrogen-dependent manner (Cappel et al., 2013; Palmisano et al., 2016). In women, increased CETP activity is associated with beneficial changes in plasma glucose after bariatric surgery (Asztalos et al., 2010). Also, plasma from women with high CETP activity had increased cholesterol efflux capacity relative to women with low CETP activity (Villard et al., 2012). In predominantly male cohorts, however, CETP activity may be associated with worsened glucose and lipid metabolism as well as increased risk of cardiovascular disease, although this remains controversial (Barter et al., 2011; Boekholdt et al., 2004; Borggreve et al., 2007; Carvalho et al., 2014; Cho et al., 2009). This suggests that CETP may have harmful effects on metabolic indices in males, and beneficial effects on metabolic indices in females. Specific mechanisms by which CETP increases risk of cardiovascular disease in males remain unknown.

Here, we determine the role of CETP in regulating TAG clearance in male mice. Previous work has shown that CETP improves reverse cholesterol transport (Tanigawa et al., 2007), but few studies have investigated the role of CETP in TAG metabolism. Previously, we demonstrated that CETP is required for estrogen to raise TAG production and to reduce liver steatosis in females (Palmisano et al., 2016). The role of CETP in regulating TAG metabolism in males is unknown. Here we test the hypothesis that CETP has sex-specific impacts on TAG metabolism in male mice.

Materials and Methods

Animals

All mouse experiments were approved under the Vanderbilt University Institutional Animal Care and Use Committee.

Mice were housed in 12 h light/dark cycles in temperature and humidity-controlled facilities with *ad libitum* access to chow diet and water. Transgenic CETP were purchased from the Jackson Laboratories (C57BL/6-Tg(CETP)UCTP20Pnu/J, Stock No: 001929). Non-transgenic littermates were used as WT controls. Male mice used in this study were aged 3–4 months old to ensure complete sexual development. All animals were sacrificed between 8 and 11 am to minimize circadian variation in gene expression. Adeno-associated virus serotype 8 (AAV8) containing short-hairpin RNA (shRNA) specific to mouse androgen receptor (AR) was purchased from Vector Biolabs (shADV-252,885, Malvern, PA) with the shRNA under control of a U6 promoter and a green fluorescence protein (GFP) reporter under control of a cytomegalovirus (CMV) promoter. AAV8 has been shown to have tropism for liver (Tenney et al., 2014). 1.8E12 GC/mouse was injected intravenously under brief isoflurane anesthesia (5%). Euthanasia was achieved using carbon dioxide and confirmed with cervical dislocation.

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 (CTAGG CCACAGAATTGAAAGATCT), Control-R (GTAGGT GGAAATTCTAGCATCATCC).

Gonadectomy and Testosterone Replacement

Animals were anesthetized under inhaled isoflurane (1–5% in 100% oxygen, 1–2 L/min). Following midline scrotal incision, gonads were externalized one by one under sterile conditions. Testicles were ligated with single interrupted 5–0 suture and excised. The incision was closed with 9 mm auto clips. Prophylactic antibiotic was given once postoperatively (ceftriaxone, 15–25 mg/kg) and analgesic was given once preoperatively and every 24 h postoperatively for 2 days (ketoprofen, 5–10 mg/kg). Mice were housed individually following surgery and allowed to recover for 2 weeks prior to study. Mice weighing less than 90% of their presurgical mass were euthanized and excluded from study. For testosterone replacement, males were gonadectomized (GDX) and allowed to recover 2 weeks. Mice were then subcutaneously injected with 300 μg/day testosterone (Cayman) in 100 μL sesame oil (Sigma-Aldrich, St. Louis, MO) for 6 days, a dose shown to restore exercise capacity in GDX male mice (Ibebunjo et al., 2011).

Lipid and Lipoprotein Analysis

Blood was collected in ethylenediaminetetraacetic acid (EDTA) containing tubes. Plasma TAG and cholesterol were measured using colorimetric kits (Infinity). Pooled plasma (100 μL) were separated into lipoproteins using fast-performance liquid chromatography (FPLC) on a Superose6 column (GE Healthcare, Chicago, IL). We define VLDL as fractions 10–17, LDL as fractions 18–28, and HDL as fractions 30–40. Liver TAG content, liver cholesterol content, and plasma testosterone levels were determined by the Vanderbilt Hormone Assay Core. For liver TAG and cholesterol content, 50–100 mg liver tissue was Folch extracted and separated by thin layer chromatography, which was then analyzed by gas chromatography with internal standards used to control for efficiency of extraction. Testosterone was measured from 25 to 35 μL plasma by radioimmunoassay using [^{3}H]-testosterone (Perkin Elmer, Waltham, MA). Plasma β-hydroxybutyrate was measured following 18 h fasting using a colorimetric kit (Cayman, Ann Arbor, MI).

In vivo TAG Clearance and Production

To measure TAG clearance, 12 h fasted mice were orally gavaged with olive oil (200 μL/mouse) and plasma TAG was measured from tail blood sampling over 5–7 h. To measure TAG production, 3 h fasted mice were given intravenous administration of Triton WR-1339 (500 mg/kg, Sigma) and plasma TAG was measured over 2 h. To measure chylomicron TAG production, 12 h fasted mice were given intraperitoneal Triton WR-1339 (500 mg/kg) 30 min prior to oral gavage with olive oil (200 μL/mouse). Plasma TAG levels were measured from tail blood sampling over 4 h.

Plasma and Lipoprotein Immunoblot

Liver tissue was snap frozen in liquid nitrogen and stored at −80°C until use. Plasma was diluted 1 to 10 in water with protease inhibitors prior to denaturation was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose. For VLDL lipoprotein composition, undiluted FPLC fractions (fractions 10–17) were denatured and separated on SDS-PAGE gels and transferred to nitrocellulose. Following overnight incubation in primary antibody (1/1000), washing, washing with 0.05% TBS-T (1X Tris buffered saline-0.05% Tween 20), and incubation in secondary antibody, immunoblots were visualized using the Odyssey infrared scanner (Licor, Lincoln, NE). Because of low protein levels of certain apolipoproteins in plasma, we relied on protein quantification (Image Studio Lite, Licor). Antibody for ApoB was purchased from Lifespan Bioscience. Antibodies to apolipoprotein A1 (ApoA1) and ApoE were purchased from Meridian Life Science (Memphis, TN). Antibodies for ApoC1 and ApoC3 were purchased from Santa Cruz (Dallas, TX). Antibodies to ApoC2 was

purchased from Abcam (Cambridge, MA). Antibodies for ApoA5 was purchased from Thermo (Waltham, MA).

Liver mRNA Quantification

Liver samples were stored in RNA-Later at 4°C overnight and then −20°C according to manufacturer's instructions (Thermo). A small piece of tissue was bead homogenized and RNA was isolated according to the manufacturer's instructions (Trizol, Qiagen, Germantown, MD). Complementary DNA was synthesized from 1 μg of mRNA (iScript, Bio-Rad, Hercules, CA). Real time reverse transcriptase polymerase chain reaction (RT-PCR) was done in triplicate from 10 ng cDNA (JumpStart Taq ReadyMix, Sigma). Primers for AR (Ar-F GGACCATGTTTTA CCCATCG, Ar-R TCGTTTCTGCTGGCACATAG) and Cyclophilin A (Ppia-F CGATGACGAGCCCTTGG, Ppia-R TCTGCTGTCTTTGGAACTTTGTC) were validated using a melting curve and annealing temperatures were optimized using gradient RT-PCR. Gene expression was quantified using efficiency corrected ΔCt method with normalization of *Ar* to *Ppia* (Pfaffl, 2001). Primer efficiency was measured independently for each RT-PCR reaction.

Statistical Analysis

All data are summarized using mean and standard error. Data from FPLC analysis are from pooled plasma samples and are thus qualitative estimates of lipoprotein distribution. Statistical tests between two groups were analyzed by unpaired Student's *t*-test. Data with more than one group were analyzed by one-way ANOVA with Bonferroni *post hoc* comparisons of selected columns. Repeated measures one-way ANOVA was used for measures of plasma TAG over time with Bonferroni post-test comparisons. Genotype effects were determined by two-way ANOVA. *p*-values <0.05 were considered statistically significant.

Results

CETP Expression Increases Plasma TAG in VLDL in Male Mice

Since transgenic expression of CETP had sex-hormone-dependent effects on TAG metabolism in females, we determined how CETP impacted TAG metabolism in males using CETP transgenic male mice and nontransgenic wild-type (WT) littermates. Expression of CETP lowered plasma cholesterol levels without changing body weight relative to WT (Fig. 1a, b). Expression of CETP increased plasma TAG over 60% in males (94.8 ± 17.8 vs 157.1 ± 26.9 mg/dL, $p < 0.001$, Fig. 1c). HDL-cholesterol was markedly reduced in CETP males (Fig. 1d, HDL fractions 32–45), which is a well-known function of CETP. We found that CETP expression resulted in a nearly twofold enrichment of TAG content of VLDL in males (Fig. 1e, fractions 10–17). CETP expression modestly increased the TAG content of HDL (Fig. 1e, fractions 32–45). Thus, transgenic expression of CETP is associated with higher plasma TAG content in VLDL in males.

CETP Raises Plasma TAG by Impairing Postprandial TAG Clearance

To determine the mechanism of how CETP increases plasma TAG and VLDL-TAG content, we measured both TAG production and postprandial TAG clearance. TAG production was similar between mice expressing CETP and WT males (Fig. 2a, b). Postprandial TAG excursion was greater in males expressing CETP relative to WT mice (Fig. 2c). Plasma area under the curve (AUC) was increased greater than 80% in CETP relative to WT mice (1271.07 mg/dL/h vs 677.25 mg/dL/h, $p < 0.05$, Fig. 2d). Since postprandial TAG levels involve a balance between gut-derived chylomicron TAG production from absorbed lipid and postprandial TAG clearance, we measured chylomicron TAG production by pretreating mice with an inhibitor of lipolysis and then giving mice an oral bolus of olive oil. Chylomicron TAG production was similar between CETP and WT males (Fig. 2e, f). This suggests that CETP expression impairs clearance of postprandial TAG in males.

CETP Expression Raises ApoB in Plasma and VLDL

To understand potential mechanisms responsible for the effect of CETP expression on postprandial TAG clearance in males, we examined the plasma apolipoproteins governing TAG clearance. Expression of CETP was associated with an over 200% increase in plasma ApoB and a 14% decrease in plasma ApoE, relative to WT males (Fig. 3a, b). Expression of CETP did not significantly alter plasma levels of other apolipoproteins involved in TAG clearance (Fig. 3a, b). Since the effect of CETP on TAG was primarily within VLDL, we next sought to understand whether the apolipoprotein composition of VLDL was altered by CETP. VLDL fractions (fractions 10 to 17) from FPLC-separated pooled plasma were separated by SDS-PAGE and then blotted for ApoB, ApoE, ApoC1, ApoC2, and ApoC3 (Fig. 3c). ApoB was qualitatively more abundant in VLDL from CETP males compared to WT males. Other apolipoproteins were qualitatively similar between

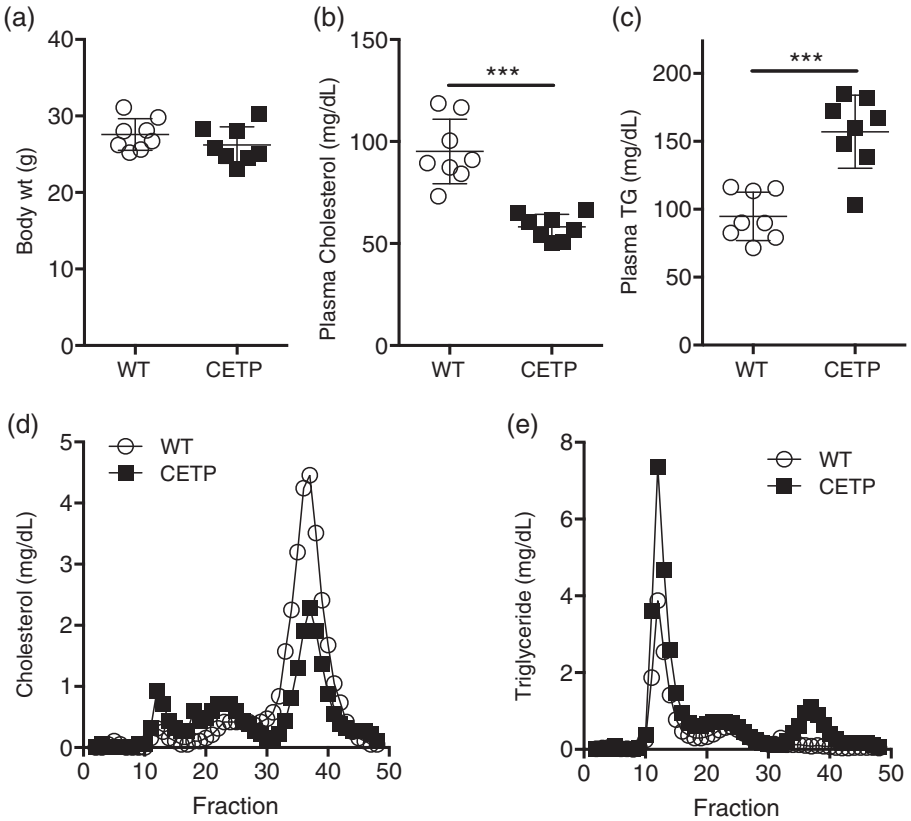

FIG 1 Plasma lipid levels in cholesteryl ester transfer protein (CETP) and wild-type (WT) males. Plasma lipids were measured in *ad lib* fed CETP and WT littermate males on chow diet. (a) CETP expression did not alter body weight ($n = 8$/group). (b) Plasma cholesterol was lower in CETP males relative to WT males ($n = 8$/group, $^{***}p < 0.001$, *t*-test). (c) Plasma triglycerides (TAG) were higher in CETP males relative to WT males ($n = 8$/group, $^{***}p < 0.001$, *t*-test). (d, e) Fast-performance liquid chromatography (FPLC) analysis of pooled plasma lipoproteins showed higher levels of very-low density lipoprotein (VLDL), especially VLDL-TAG in CETP males relative to WT males and lower levels of high-density lipoprotein (HDL) cholesterol in CETP males than WT males

CETP males and WT males. ApoE was not detected in VLDL fractions from CETP or WT males. Thus, the decrease in plasma ApoE is likely due to a decrease in ApoE in other lipoprotein classes, which has been observed and described previously in HDL (Zak et al., 2002). Additionally, although prior work suggested that expression of CETP was associated with altered liver lipid content and differences in liver fat oxidation in females (Palmisano et al., 2016), we did not observe changes in liver lipid content or liver fat oxidation in CETP males relative to WT males (data not shown).

CETP Requires Gonadal Hormones to Raise Plasma TAG in Males

Sex steroids were required for CETP to raise plasma TAG in females (Palmisano et al., 2016). To further examine hormonal mechanisms by which CETP impairs TAG clearance in males, we determined if sex steroids were required for the elevated plasma TAG seen with CETP expression males by measuring plasma lipids after GDX in WT and CETP mice. Males with intact gonadal hormones had similar levels of plasma testosterone levels in both CETP and WT males (Fig. 4a). Gonadectomy reduced plasma testosterone levels greater than 1000-fold in both groups (Fig. 4a). Body weight was not different between GDX WT or GDX CETP males (Fig. 4b). With GDX, CETP decreased plasma cholesterol by approximately 25% relative to WT (52.45 vs 69.97 mg/dL, GDX CETP vs GDX WT, Fig. 4c, $^{*}p < 0.05$). The magnitude of this cholesterol reduction is similar to males with intact gonadal hormones. Additionally, the cholesterol reduction is predominantly within HDL, which is also similar to males with intact gonadal hormones (Fig. 4e, similar to Fig. 1d). In the absence of gonadal hormones, however, plasma TAG levels were similar between CETP and WT males (80.69 vs 67.79 mg/dL, GDX CETP vs GDX WT, Fig. 4d, p = ns). Additionally, VLDL-TAG content was similar in GDX CETP and WT males (Fig. 4f). Furthermore, in the absence of gonadal hormones, postprandial TAG excursion was similar in CETP and WT males (Fig. 4g, h). Thus, in the absence of gonadal hormones, CETP expression fails to

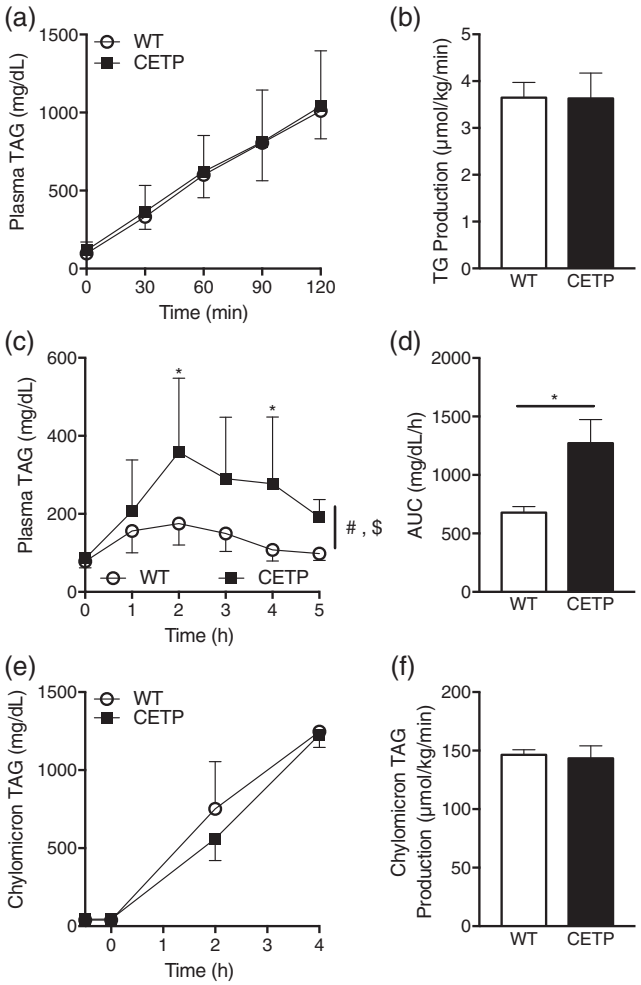

FIG 2 Triglyceride (TAG) metabolism in cholesteryl ester transfer protein (CETP) and wild-type (WT) males. (a, b) Very-low density lipoprotein (VLDL)-TAG production was measured in 3-h fasted WT and CETP males after an intravenous bolus of triton WR-1339 (500 mg/kg). VLDL-TAG over time (a) and VLDL-TAG production rate (b) was not different between CETP and WT male mice. (c, d) following a 12 h overnight fast, CETP and WT male mice were orally gavaged with olive oil (200 μL/mouse). Postprandial TAG excursion was measured over 5 h. CETP impaired postprandial TAG excursion over time (c) and postprandial TAG area under the curve (AUC) (d). Repeated measures two-way ANOVA #$p < 0.05$ for genotype (CETP vs WT), \$$p < 0.05$ for interaction (genotype vs time), *$p < 0.05$ (post-hoc comparison CETP vs WT, c) *$p < 0.05$ (*t*-test, d). (e, f) chylomicron TAG production was measured in 12-h fasted WT and CETP males pretreated with triton WR-1339 (500 mg/kg) 30 min prior to oral olive oil gavage (200 μL/mouse). Plasma chylomicron TAG levels over time (repeated-measures ANOVA, E) and production rate (*t*-test, f) were similar between CETP and WT males

alter TAG metabolism, but still lowers total plasma cholesterol and reduces HDL cholesterol content.

We next examined whether gonadal hormones were also required for CETP to alter plasma apolipoprotein levels, as seen in male mice with intact gonadal hormones. In the absence of gonadal hormones, plasma levels of ApoB and ApoE were similar in CETP and WT males (Fig. 5a, b). Thus, gonadal hormones are associated with CETP-mediated changes in plasma levels of some apolipoproteins.

Testosterone Treatment Restores the Hypertriglyceridemic Effect of CETP in GDX Mice

To determine if testosterone was sufficient to restore the hypertriglyceridemic effect of CETP, we treated GDX mice with testosterone. Following GDX, WT and CETP males were given testosterone (300 μg/day) for 6 days prior to study, a dose that restores exercise capacity in GDX male mice (Ibebunjo et al., 2011). Following testosterone treatment, body weight, and body weight gain were similar in WT and CETP mice (Fig. 6a, b). Plasma testosterone levels were similar in both WT and CETP mice after testosterone treatment (Fig. 6c), albeit 10–20 times greater than endogenous levels (Fig. 6c vs Fig. 4a). CETP lowered plasma cholesterol 40% relative to WT following testosterone treatment (70.85 vs 119.07 mg/dL, Fig. 6d, ***$p < 0.001$). With testosterone treatment, CETP increased plasma TAG nearly 40% (166.27 vs 120.25 mg/dL, Fig. 6e, *$p < 0.05$). Similar to plasma changes, CETP reduced cholesterol content of HDL (Fig. 6f) and increased TAG content of VLDL relative to WT males after testosterone treatment (Fig. 6g). Taken together, these data suggest testosterone is sufficient for CETP to raise plasma TAG in the setting of GDX.

Androgen Receptor Is Required for the Hypertriglyceridemic Effect of CETP

Since testosterone was sufficient to restore the hypertriglyceridemic effect of CETP, we sought to understand the molecular mechanism of how CETP raises plasma TAG by knocking down expression of AR. Using a liver tropic adeno-associated virus (AAV8) (Tenney et al., 2014), we used a short-hairpin RNA (shRNA) to knockdown androgen receptor (shAR) ~90% relative to control mice transduced with a scrambled shRNA (shSCR) (Fig. 7a). AR knockdown did not alter body weight or cause weight gain in WT or CETP mice (Fig. 7b, c). Similar to both intact and GDX males, CETP reduced plasma cholesterol levels 40–70% (22.61 vs 77.18 mg/dL for shSCR treated mice; 57.88 vs 97.83 mg/dL for shAR treated mice, Fig. 7d, ***$p < 0.001$). With AR knockdown, however, CETP failed to increase plasma TAG relative to WT (153.05 vs 162.53 mg/dL, Fig. 7e, p = ns). CETP expression was still associated with increased plasma TAG in control-treated mice (345.01 vs 163.73 mg/dL, Fig. 7e, ***$p < 0.001$). Also similar to intact and GDX males, CETP reduced HDL cholesterol content but failed to alter TAG content of VLDL with AR knockdown (Fig. 7f, g).

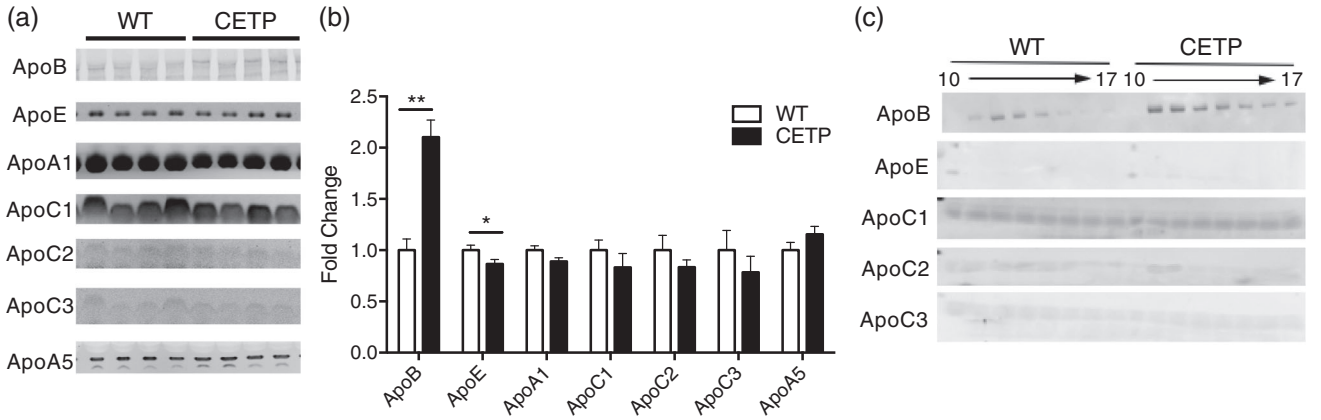

FIG 3 Plasma and very-low density lipoprotein (VLDL) levels of Apolipoproteins in cholesteryl ester transfer protein (CETP) and WT males. (a, b) Western blot (a) and quantification (b) of plasma lipoproteins from wild-type (WT) and CETP male mice. CETP increased expression of ApoB and reduced expression of ApoE. $^{**}p < 0.01$, $^{*}p < 0.05$ (*t*-test). (c) Apolipoprotein composition of VLDL fractions (fractions 10–17) from fast-performance liquid chromatography (FPLC) separated pooled plasma from WT and CETP male mice. Diluted plasma was run in lane 10 for ApoE, ApoC1, ApoC2, and ApoC3 due to low-intensity signal for comparison

Liver expression of GFP, a marker of vector transduction, was similar in both WT and CETP males (data not shown). Thus, CETP requires liver AR raise plasma TAG. Taken together, these data support a novel function of CETP that impairs postprandial plasma TAG clearance in males *via* a novel pathway involving testosterone signaling to AR.

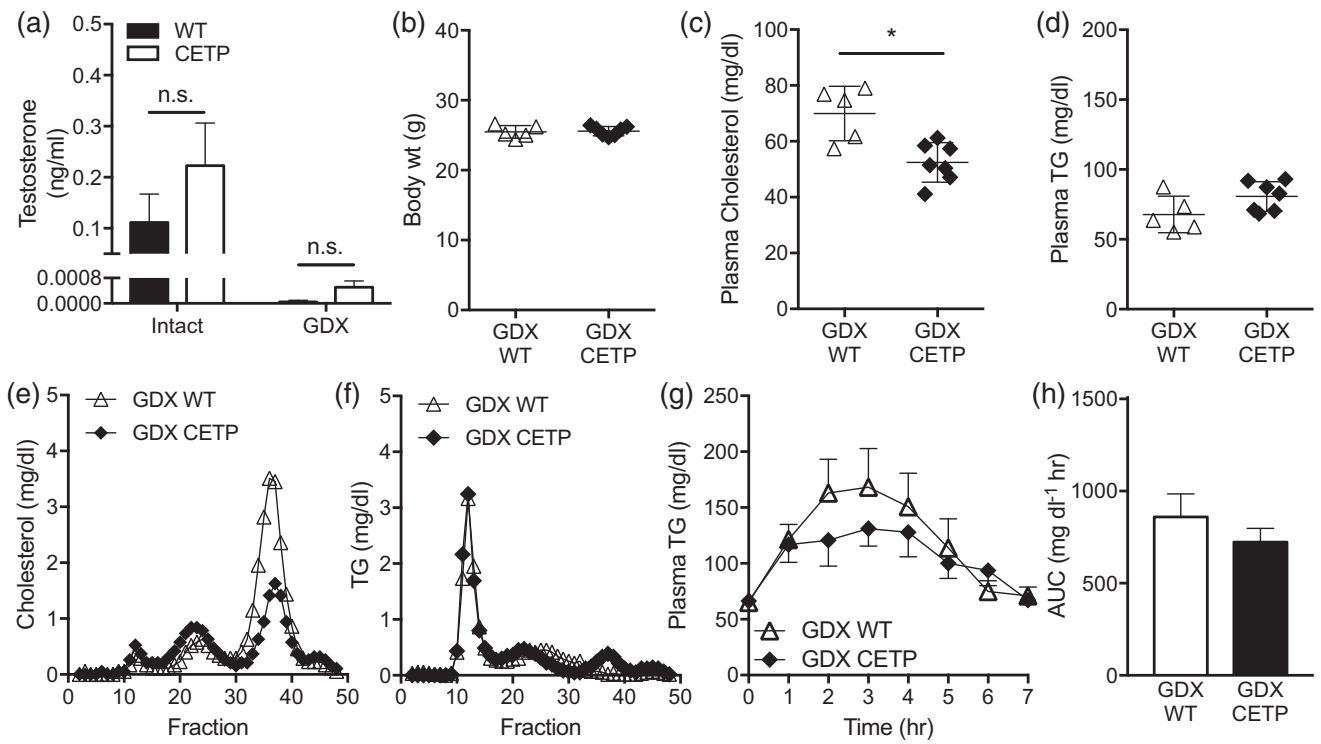

FIG 4 Effect of gonadectomy on triglyceride (TAG) metabolism in cholesteryl ester transfer protein (CETP) and wild-type (WT) males. CETP and WT male littermates were gonadectomized (GDX) 2 weeks prior to study (n = 5–7/group). (a) CETP did not alter plasma testosterone levels in intact males compared to WT males. GDX reduced testosterone levels over 1000-fold. (b) Body weight was no different between GDX CETP and GDX WT males. (c) Plasma cholesterol was lower in GDX CETP males relative to GDX WT males. $^{*}p < 0.05$ (*t*-test) (d) plasma TAG was not significantly different in GDX CETP males relative to GDX WT males. (e, f) Fast-performance liquid chromatography (FPLC) analysis of pooled plasma lipoproteins showed lower levels of high-density lipoprotein (HDL) cholesterol, but unchanged levels of very-low density lipoprotein (VLDL)-TAG in GDX CETP males relative to GDX WT males. (g, h) postprandial TAG excursion was measured over 7 h following oral gavage with olive oil (200 μL/mouse) in 12-h overnight fasted GDX CETP and WT mice (n = 7/group). CETP did not impair postprandial TAG excursion over time (g) or postprandial TAG area under the curve (AUC) (h) in the absence of gonadal hormones

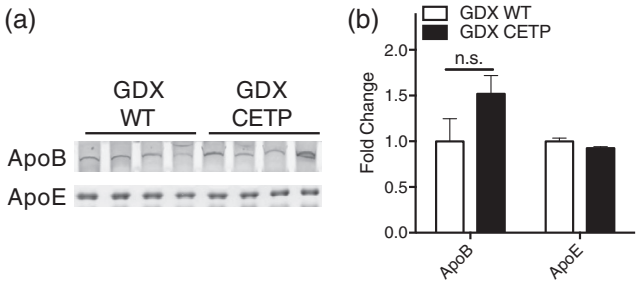

FIG 5 Effect of gonadectomy on plasma levels of apolipoproteins B and E in cholesteryl ester transfer protein (CETP) and wild-type (WT) males. (a, b) plasma lipoprotein expression was measured by western blotting (a) and quantified (b) in GDX WT and GDX CETP male mice

Discussion

Currently, mechanisms governing sex differences in lipid metabolism remain incompletely understood. In this study, we demonstrate a novel male-specific pathway whereby CETP raises plasma TAG by impairing postprandial TAG clearance. We demonstrate that the molecular mechanism of this pathway involves testosterone signaling to AR in the liver. Gonadal hormones were required for CETP to raise plasma TAG, impair postprandial TAG clearance. Testosterone treatment was sufficient to restore these effects. Lastly, knockdown of AR prevented CETP from raising plasma TAG, even in the presence of endogenous gonadal hormones in males. Thus, the molecular mechanism whereby CETP impairs plasma TAG clearance involves testosterone signaling to liver AR.

This report highlights several roles for CETP in sex differences in lipid metabolism. Previously, we showed that CETP raises plasma TAG in females by increasing VLDL-TAG production in response to estrogen with estrogen in females by increasing VLDL-TAG production (Palmisano et al., 2016). The molecular mechanism involved nuclear receptors Small Heterodimer Partner and Estrogen Receptor α (Palmisano et al., 2016). Our previous study and others have shown that CETP expression impairs plasma TAG

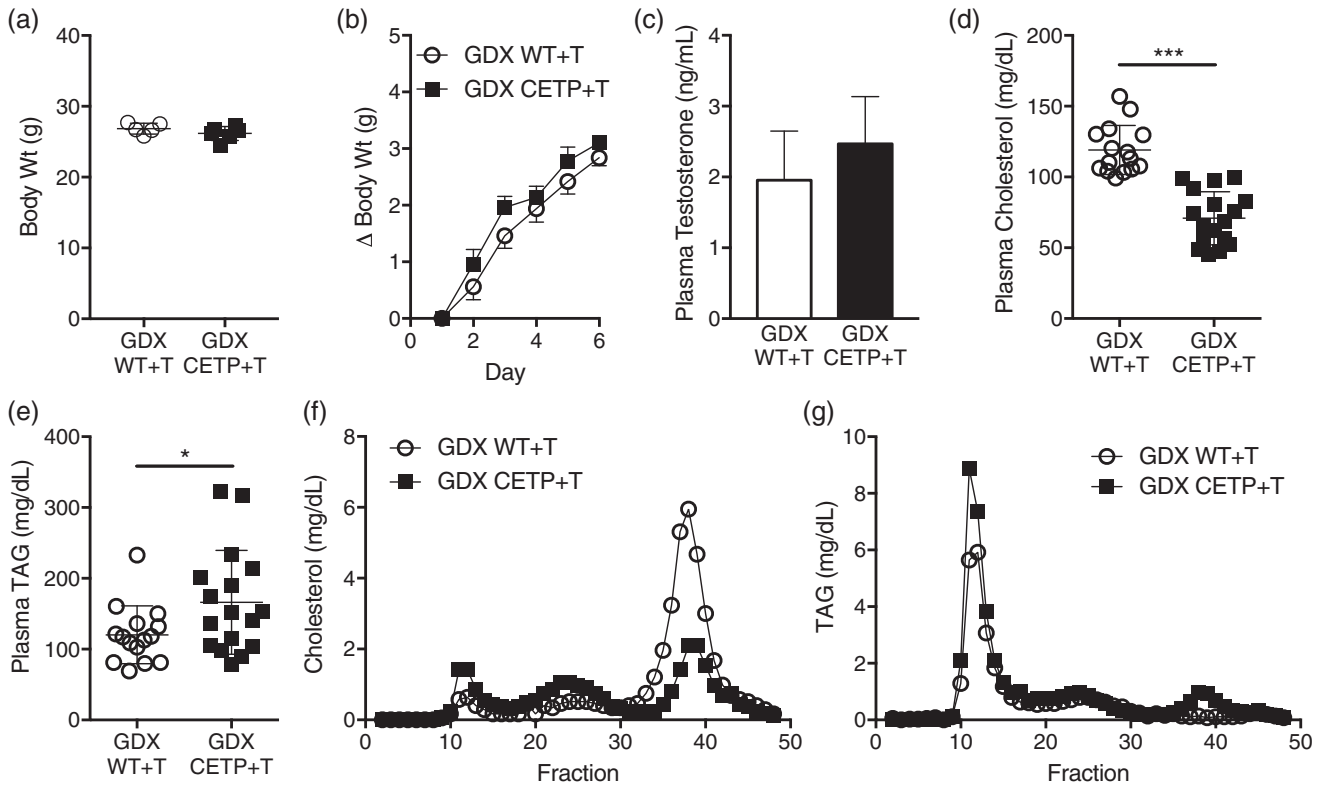

FIG 6 Effect of testosterone replacement on plasma lipids in gonadectomized cholesteryl ester transfer protein (CETP) and wild-type (WT) males. WT and CETP males were gonadectomized 2 weeks prior to study and given testosterone (300 μg/day) for 6 days prior to study. (a, b) body weight (a) and body weight gain (b) with testosterone replacement were similar between CETP and WT males (n = 5-6/group). (c) Plasma testosterone levels were similar between CETP and WT males (n = 11–12/group). CETP expression did not alter body weight. (d) Plasma cholesterol was lower in CETP males relative to WT males (n = 16-17/group ***$p < 0.001$, t-test). (e) Plasma triglyceridess (TAG) were higher in CETP males relative to WT males (n = 16-17/group, *$p < 0.01$, t-test). (f, g) Fast-performance liquid chromatography (FPLC) analysis of pooled plasma lipoproteins showed higher levels of very-low density lipoprotein (VLDL), especially VLDL-TAG in CETP males relative to WT males and lower levels of high-density lipoprotein (HDL) cholesterol in CETP males than WT males

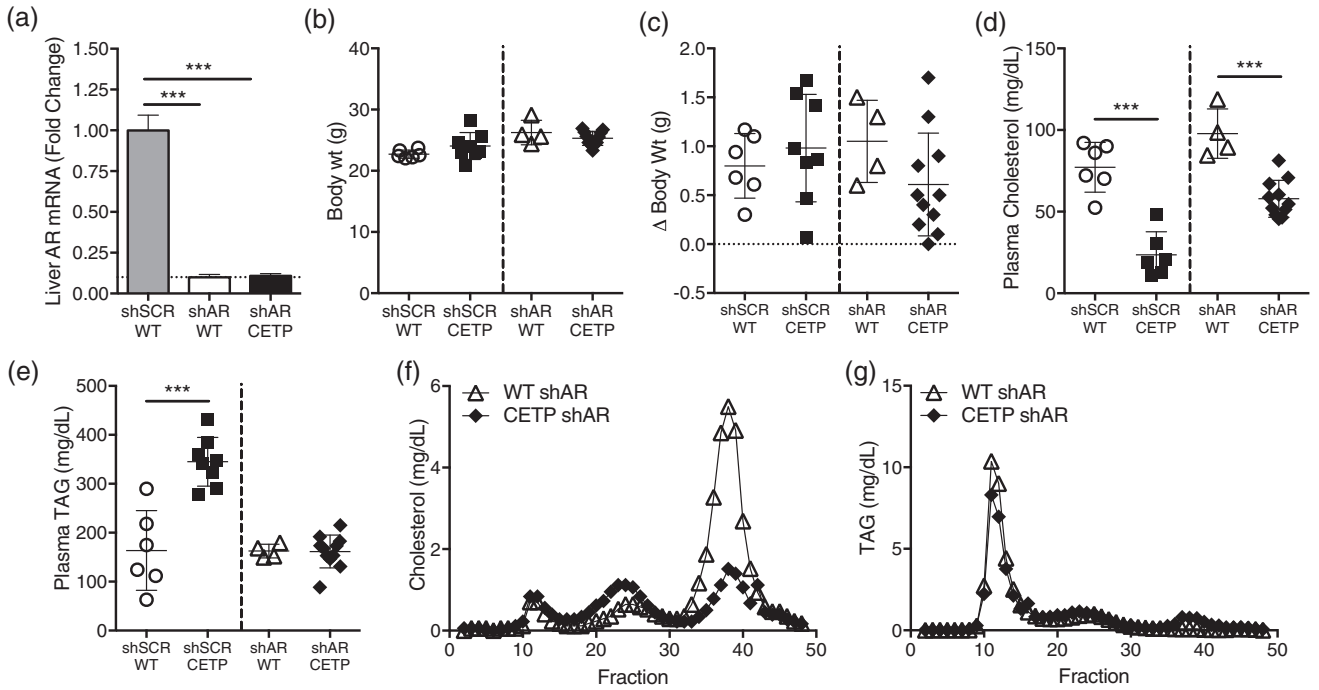

FIG 7 Effect of androgen receptor knockdown on plasma lipids in cholesteryl ester transfer protein (CETP) and wild-type (WT) males. (a) Androgen receptor mRNA expression was knocked down in WT and CETP males using a short hairpin RNA (shRNA) *via* intravenous injection of adeno-associated virus serotype 8 (AAV8) 2 weeks prior to study. Liver mRNA expression of androgen receptor was decreased ~90% in both WT and CETP males ($n = 4$–11/group, scrambled shRNA [shSCR] vs androgen receptor shRNA, shAR, ***$p < 0.001$, one-way ANOVA with *post hoc* comparisons). (b, c) body weight (b) and body weight change (c) were not different between WT and CETP males treated with shSCR or shAR ($n = 4$–11/group, *t*-test). (d) Plasma cholesterol was lower in CETP males relative to WT males in both shSCR and shAR treated mice ($n = 4$–11/group ***$p < 0.001$, *t*-test). (e) CETP was associated with higher plasma triglycerides (TAG) relative to WT males in shSCR treated mice ($n = 6$–8/group, ***$p < 0.001$, *t*-test). Plasma TAG were similar between shAR treated CETP and WT males ($n = 4$–11/group, *t*-test). (f, g) Fast-performance liquid chromatography (FPLC) analysis of pooled plasma lipoproteins showed lower levels of high-density lipoprotein (HDL) cholesterol in but no change in very-low density lipoprotein (VLDL)-TAG in CETP males relative to WT males. Vertical dashed lines indicate separate cohorts of mice

clearance in females, but the mechanism for this observation was unknown (Palmisano et al., 2016; Salerno et al., 2009). Our previous work suggests that the impact of CETP was independent of liver Estrogen Receptor α (Palmisano et al., 2016). Additionally, the impact of CETP on TAG clearance in males has been under studied as a majority of studies focus on the impact of CETP on HDL cholesterol levels. Here, we show that in males, CETP requires liver AR, to alter plasma TAG metabolism. Thus, CETP expression increases plasma TAG in both males and females, but through distinct, sex-specific mechanisms involving a novel network of nuclear hormone receptors. Another important observation in this study is that the hypocholesterolemic effect of CETP is distinct from the hypertriglyceridemic effect of CETP. CETP reduces HDL cholesterol enriching HDL with TAG, which promotes HDL clearance (Lamarche et al., 1999). We show that sex hormones were required for the hypertriglyceridemic effect of CETP, but the cholesterol lowering effect of CETP was independent of sex hormone signaling. Future work is necessary to isolate the mechanisms of these discrete functions of CETP. Although we show that liver AR is required for CETP-mediated effects on plasma TAG, it remains to be determined whether this is a direct liver tissue effect or an indirect effect of CETP function in plasma or in extrahepatic tissues and future work is needed to work out this novel signaling network. This work supports an important role for CETP in both AR signaling and TAG metabolism in males.

Here, we demonstrate that CETP creates a gain-of-function response to testosterone, leading to impaired postprandial TAG clearance. In CETP males, GDX reduced plasma TAG to levels seen in WT males. In WT males, however, GDX did not substantially alter plasma TAG in WT males. This indicates that CETP does not augment an existing pathway regulating plasma TAG. Rather, CETP expression causes a gain-of-function response to testosterone by impairing postprandial TAG clearance. This impaired postprandial TAG clearance is lost with GDX and restored with testosterone replacement. This testosterone-mediated pathway is lost when liver AR expression is knocked down, indicating liver AR is the mechanistic link

between CETP and impaired postprandial TAG clearance in males. A counter to this hypothesis is that the effect of GDX supersedes the effect of CETP on increasing plasma TAG. However, others have shown that TAG are able to be further decreased (Chiba et al., 2014) or increased (Makino et al., 2012) in the setting of GDX. Thus, GDX is not such a severe metabolic insult to preclude mechanistic study of TAG metabolism. Although we show that liver AR is the mediator of male-specific impairment of postprandial TAG clearance, the target or targets of AR responsible for this impairment remain to be discovered. Plasma ApoE levels were lower in CETP males, but the magnitude of this ApoE reduction is unlikely to be physiologically relevant. However, further study is needed to draw specific conclusions on the liver AR targets responsible for CETP-mediated impairment of postprandial TAG clearance. This gain-of-function response to testosterone in male mice expressing CETP mirrors the gain-of-function response to estrogen signaling in female mice expressing CETP, but further work is needed to understand whether a common pathway regulates estrogen receptor and AR signaling similarly in mice expressing CETP (Palmisano et al., 2016).

The work presented here adds to a clinical story that CETP may be proatherogenic in males since we found that CETP expression impairs TAG clearance. Impaired TAG clearance is an important risk factor for atherosclerosis in men (Boquist et al., 1999; Carstensen et al., 2004; Ginsberg et al., 1995; Groot et al., 1991; Karpe et al., 1994; Sharrett et al., 1995). The role of CETP in atherosclerosis in humans is controversial. While some studies suggest a positive correlation between CETP activity and risk of cardiovascular disease (Boekholdt et al., 2004; Borggreve et al., 2007; Carvalho et al., 2014; Cho et al., 2009), other studies suggest a neutral or inverse association with cardiovascular disease (Duwensee et al., 2010; Goto et al., 2001; Kappelle et al., 2011; Khera et al., 2010; Marschang et al., 2006; Ritsch et al., 2010; Robins et al., 2013; Vasan et al., 2009; Zeller et al., 2007). Interestingly, CETP activity seems to increase risk of cardiovascular disease when TAG levels are high (Boekholdt et al., 2004). This may suggest that CETP is proatherogenic only in the setting of hypertriglyceridemia because the association between CETP and cardiovascular disease was lost when TAG levels were low. Interestingly, only one (Group et al., 2017) of four major phase 3 randomized controlled trials of CETP inhibitors reduced cardiovascular disease outcomes (Barter et al., 2007; Group et al., 2017; Lincoff et al., 2017; Schwartz et al., 2012). Subgroup analysis revealed that CETP inhibition by anacetrapib was significant only in males and only in patients with hypertriglyceridemia (TAG >151 mg/dL) (Group et al., 2017). This supports the concept that CETP may have sex-specific function in humans. This also supports the hypothesis that CETP inhibition may reduce cardiovascular disease outcomes by reducing TAG as well as LDL in humans. Indeed, CETP inhibition has been previously shown to reduce plasma TAG levels by increasing apoB and TAG clearance (Diffenderfer et al., 2012; Millar et al., 2006, 2015). Thus, future work aimed at understanding how CETP alters TAG metabolism may lead to more targeted therapies that reduce risk of cardiovascular disease.

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Conflict of Interest J.C.N. is currently a Novo Nordisk Inc. employee but was at Vanderbilt during the time the studies were performed. All other authors declare that they have no conflicts of interest.

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