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Both full length-CETP and exon 9-deleted CETP promote triacylglycerol storage in cultured hepatocytes

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Abstract

We previously reported that overexpression of full-length cholesteryl ester transfer protein (FL-CETP), but not its exon 9-deleted variant (ΔE9-CETP), in an adipose cell line reduces their triacylglycerol (TAG) content. This provided mechanistic insight into several in vivo studies where FL-CETP levels are inversely correlated with adiposity. However, increased FL-CETP is also associated with elevated hepatic lipids, suggesting that the effect of CETP on cellular lipid metabolism may be tissue-specific. Here, we directly investigated the role of FL-CETP and ΔE9-CETP in hepatic lipid metabolism. FL- or ΔE9-CETP was overexpressed in HepG2-C3A by adenovirus transduction. Overexpression of either FL or ΔE9-CETP in hepatocytes increased cellular TAG mass by 25% but reduced TAG secretion. This cellular TAG was contained in larger and more numerous lipid droplets. Analysis of TAG synthetic and catabolic pathways showed that this elevated TAG content was due to increased incorporation of fatty acid into TAG (24%), and higher *de novo* synthesis of fatty acid (50%) and TAG from acetate (40%). siRNA knockdown of CETP had the opposite effect on TAG synthesis and lipogenesis, and decreased cellular TAG. This novel increase in cellular TAG by FL-CETP overexpression was reproduced in Caco-2 intestinal epithelial cells. We conclude that, unlike that seen in adipocyte cells, overexpression of either CETP isoform in lipoprotein-secreting cells promotes the accumulation of TAG. These data suggest that the in vivo correlation between CETP levels and hepatic steatosis can be explained, in part, by a direct effect of CETP on hepatocyte cellular metabolism.

Keywords

cholesteryl ester transfer protein; isoforms; lipid metabolism; triacylglycerol; cellular lipid homeostasis; hepatocyte

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

INTRODUCTION

Cholesteryl ester transfer protein (CETP) mediates the transfer of cholesteryl ester (CE) and triacylglycerol (TAG) among plasma lipoproteins (Morton, 1990). CETP is widely expressed in a variety of tissues, including liver, adipose tissue, small intestine, and adrenal gland (Drayna et al., 1987; Jiang et al., 1991; Tall, 1995). Two major forms of CETP exist in these tissues - full-length CETP (FL-CETP) containing 476 amino acids, and exon 9 deleted CETP (ΔE9-CETP), a smaller form lacking 60 amino acids due to alternative splicing (Inazu et al., 1992). Most FL-CETP is secreted after being synthesized. In contrast, ΔE9-deleted CETP remains intracellular (Inazu et al., 1992). The ratio of FL- to ΔE9-CETP mRNA is altered by diets and varies among the tissues (Yang et al., 1996). In some cells, ΔE9-deleted CETP mRNA accounts for more than half of total CETP mRNA (Inazu et al., 1992).

The central role of CETP in modulating plasma lipoprotein metabolism and altering HDL levels in humans has been extensively studied (Klerkx et al., 2006). Beyond that, we have shown that CETP also impacts cellular lipid homeostasis. In SW872 adipocytes, overexpression of FL-CETP impairs TAG storage capacity (Izem et al., 2015). In contrast, ΔE9-deleted CETP overexpression stimulates TAG synthesis and storage (Izem et al., 2020). Consistent with a role for CETP in adipocyte lipid storage, several studies in animals have shown an association between increased FL-CETP expression and decreased adiposity. Human CETP transgenic mice have reduced adiposity, whereas inhibition of CETP in hamsters increased their adiposity (Raposo et al., 2021). Similarly, mice with adipose tissue-specific human CETP expression develop smaller adipocytes containing less TAG and cholesterol and reduced expression of key lipogenic genes (Zhou et al., 2006). CETP expression in hypertriglyceridemic mice alleviates obesity and decreased adipocyte size (Salerno et al., 2007). And in humans, a CETP gene variant that affects the coding sequence of both FL- and ΔE9-CETP is associated with increased adiposity (Terán-García et al., 2008).

However, several in vivo studies suggest that the impact of CETP on lipid homeostasis may be different in other tissues. In humans, increased CETP activity has a positive association with hepatic lipid accumulation (Lucero et al., 2011; Fadaei et al., 2018). Transgenic mice expressing cynomolgus monkey FL-CETP exhibit elevated post-prandial hypertriglyceridemia and develop hepatic lipid accumulation on a normal chow diet (Blake et al., 1994; Salerno et al., 2009). And in hamsters, human FL-CETP expression increases hepatic cholesterol without altering plasma cholesterol levels (Morton et al., 2021). Conversely, in rabbits, an anti-CETP vaccine reduces atherosclerosis and hepatic steatosis (Liaw et al., 2014). Overall, these studies suggest that CETP promotes hepatic lipid accumulation.

Taken together, the above data suggest that the impact of CETP on cellular lipid homeostasis may be different in cells whose primary function is to store lipid (adipocytes) versus those involved in lipoprotein assembly and secretion (hepatocytes, intestinal epithelium). While the impact of CETP on lipid homeostasis has been directly explored in adipocytes (Izem & Morton, 2007; Izem et al., 2015; Izem et al., 2020), such information is lacking in other cell types. In this study, we directly examined the impact of CETP overexpression in cultured

hepatocytes and intestinal epithelial cells on lipid content and on the activities of metabolic pathways such as fatty acid uptake, lipogenesis, lipid secretion, and lipolysis that control cellular lipid homeostasis.

METHODS and MATERIALS

Materials

[9,10-^{3}H(N)] oleic acid and [1,2-^{14}C] sodium acetate were purchased from Perkin-Elmer Life Sciences (Waltham, MA). Stock 5 mM ^{3}H-oleate/BSA and unlabeled oleate/BSA (6:1 mol ratio of oleate to BSA) were prepared as previously described (Clevidence et al., 1984). The mouse monoclonal antibody against human CETP, TP2, was purchased from the Ottawa Heart Institute (Ottawa, Ontario, Canada). Penicillin, streptomycin, BSA, and sodium oleate were from Sigma-Aldrich Corp. (St. Louis, MO). CI-1011 was a gift from Pfizer.

CETP overexpression and knockdown in HepG2/C3A cells

Human hepatocellular carcinoma C3A cells (CRL-10741), a HepG2 subline, were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in EMEM containing 10% FBS (Atlas Biological, Fort Collins, CO) and 50 μg/ml penicillin-streptomycin in 5% carbon dioxide, 95% air at 37°C. The human colon adenocarcinoma Caco-2 (HTB-37), was purchased from American Type Culture Collection. Caco-2 cells were cultured in DMEM/F-12 (3:1) containing 10% FBS plus antibiotics.

In HepG2/C3A cells, transient CETP expression was achieved by transduction with recombinant adenoviruses (type 5, E1 and E3 regions deleted). Adenovirus constructs containing FL-CETP or ΔE9-CETP were prepared as previously described (Izem et al., 2020). For transduction, cells at 70% confluence were incubated in media plus adenovirus (30 pfu/cell). After 6 h, cells were switched to media containing serum and incubated overnight before use in experiments (Liu et al., 2019). For CETP knockdown, C3A cells at 70% confluence were transfected with 20 nM control siRNA (AM4611, ThermoFisher Scientific Inc., Waltham, MA) or CETP siRNA (s2933, ThermoFisher Scientific Inc.) using Lipofectamine 3000 following the manufacturer's directions (Life Technologies, Grand Island, NY). Cells were switched to media containing serum and incubated overnight before use in experiments.

Transient FL-CETP expression in Caco-2 cells

A pCMV-Sport6 construct containing the open reading frame of human FL-CETP was purchased from Open Biosystems (Huntsville, AL). The EcoRI / NotI fragment containing CETP was subcloned into pcDNA3. Cells at 70% confluence were transfected with 10 μg native pCDNA3 or pCDNA3-FL-CETP plasmid using Lipofectamine 2000 transfection reagent (Life Technologies) following the manufacturer's instructions (Izem et al., 2020). After 5 h, media containing 10% FBS was added and cells were allowed to recover overnight before use in experiments.

Assay of cellular and secreted CETP

Transfected or transduced cells were incubated in serum-free media for 48 h. Conditioned media was centrifuged (10 min, 16,000 × g, 25°C) to remove debris. Cells were washed three times with PBS, harvested in RIPA buffer, and incubated on ice for 30 min with frequent mixing. Lysates were centrifuged at 10,000 g for 10 min and their protein content was determined by a BCA Protein Assay kit (ThermoFisher Scientific Inc.). HepG2/C3A conditioned media was concentrated 10-fold by cold acetone precipitation after spinning down the debris. Western blots for CETP (TP2 antibody) were performed as previously described (Izem et al., 2015). ApoAI (Sigma-Aldrich) was used as a loading control (Morton et al., 2019).

Conditioned media was also assayed for CETP transfer activity with ^{3}H-CE LDL (donor) and HDL (acceptor) as previously described (Morton & Zilversmit, 1981).

Cellular TAG mass

Transfected or transduced cells were incubated in serum-free media without or with 100 μM oleate/BSA for 48 h. The intracellular TAG mass was measured with a commercial kit (K622, BioVision, Milpitas, CA).

Lipid droplet staining

Cells were cultured on glass coverslips in 12-well plates. After 70% confluence, the cells were incubated with 100 μM oleate/BSA for 48 h. Cells were fixed using 4% formaldehyde, then stained for 15 min with 200 ng/ml BODIPY 558/568 in PBS. The coverslips were applied using 4',6-diamidino-2-phenylindole (DAPI)-containing mounting solution (Vectashield, Burlingame, CA). Cells were visualized by fluorescence microscopy (Olympus IX51, Center Valley, PA), and lipid droplet size and number were determined by ImageJ using software default parameters (National Institutes of Health, (https://imagej.nih.gov/ij/)). Lipid droplets in eight fields containing 131–150 cells/field for each cell type were analyzed.

^{3}H-oleate incorporation into cellular and secreted lipids

Cells were washed with PBS and incubated in serum-free DMEM with 100 μM ^{3}H-oleate/BSA for 48 h. Conditioned cell media was centrifuged (10 min, 16,000 × g, 25°C) to remove cell debris. Culture plates were kept on ice until cells were scraped. Lipids in the cells and media were extracted (Bligh & Dyer, 1959) and separated by thin layer chromatography in a system of hexanes-diethyl ether-acetic acid (80:20:1, v/v). After exposure to iodine vapors, lipid fractions were identified based on comigration with authentic lipid standards (Nu-chek Prep Inc., Waterville, MN; and Avanti Polar Lipids Inc., Alabaster, AL). Radioactivity was determined by scintillation counting (Izem et al., 2015). Cell protein content was quantified by a BCA Protein Assay (ThermoFisher Scientific Inc.)

TAG synthetic rates

Cells were cultured in growth media containing 100 μM unlabeled oleate/BSA for 24 h to initiate lipid droplet formation. Cells were then washed with media and incubated in media containing 100 μM ^{3}H-oleate/BSA. Synthesis was stopped by removing the media

and washing cells with cold PBS. Culture plates were kept on ice until cells were scraped. Cellular lipids were extracted and separated by thin layer chromatography (Izem et al., 2015). TAG bands were scraped and ^{3}H quantified by scintillation counting.

TAG hydrolysis

To determine the rate of TAG turnover, cells were incubated in growth media supplemented with 200 μM ^{3}H-oleate/BSA for 24 h to label the cellular TAG pool. The cells were washed with warm media, then either harvested (t = 0) or incubated for the indicated times in the presence of 10 μM triacsin C (Sigma-Aldrich Corp.), a fatty acyl-CoA synthetase inhibitor (Tomoda et al., 1987). Cells were harvested, and lipids extracted and fractionated by thin layer chromatography as described above (Izem et al., 2015).

De novo lipogenesis

De novo synthesis of fatty acid and TAG was quantified from the incorporation of ^{14}C-acetate into these lipids (Liu et al., 2019). Cells were incubated in serum-free DMEM with 30 μM sodium ^{14}C-acetate for 4 hr. Lipids in the cells were extracted and fractionated by thin layer chromatography (Izem et al., 2015).

Fatty acid uptake

Cells were incubated in serum-free DMEM containing 200 μM ^{3}H-oleate/BSA for the indicated time. At each time point, the cells were washed with cold PBS, scraped from the dish, washed three additional times with cold PBS, and solubilized in 0.1 N NaOH. The ^{3}H-content was determined by scintillation counting (Izem et al., 2015).

Real-time quantitative PCR

Total RNA was extracted using Trizol reagent (ThermoFisher Scientific Inc.), Waltham, MA) or an RNeasy Mini Kit from Qiagen (Germantown, MD) following the manufacturers' protocols. cDNA was generated from 5 μg total RNA using a High Capacity RNA-to-cDNA kit (ThermoFisher Scientific Inc.). Real-time quantitative PCR (qPCR) analysis was performed using TaqMan gene expression primer/probe sets and TaqMan Universal Master Mix from Life Technologies. mRNA values were normalized to GAPDH mRNA (Barber et al., 2005). Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and reported relative to control cells.

Statistical analysis

Statistical analysis between two groups was performed by unpaired t-test. For multiple comparison between groups, statistical analysis was performed by one-way ANOVA with Dunnett's post-comparison test or two-way ANOVA with Tukey's post-comparison test to determine adjusted P values (Prism, GraphPad Software, La Jolla, CA). In all cases, P values < 0.05 were considered statistically significant.

RESULTS

CETP overexpression in HepG2/C3A cells

To assess the impact of CETP on lipid metabolism in liver cells, we over expressed CETP in a human hepatocyte cell line, HepG2/C3A. Cultured hepatocytes such as HepG2/C3A have very low CETP expression (~10% of liver). This likely reflects from the rapid loss of CCAAT/enhancer-binding protein expression and subsequent loss of CETP mRNA that occurs early in the culture of primary hepatocytes. Thus, HepG2/C3A cells reflect a state of significant CETP deficiency. Our model of over expression likely corrects this deficiency and overexpresses CETP compared to native hepatocytes.

HepG2/C3A cells were transduced with adenoviruses expressing with FL-CETP or its shorter ΔE9-CETP isoform. CETP mRNA levels measured by a probe targeting a shared region of FL- and ΔE9-CETP mRNA show that at a dosage of 30 pfu/cell, transduction of the two CETP isoforms induced similar levels of CETP mRNA (Figure 1A). CETP protein was measurable only in the conditioned media from the cells overexpressing FL-CETP (Figure 1B), indicating that CETP is properly synthesized and secreted into the media. Both CETP isoforms were detected in the cell lysates of transduced cells (Figure 1B). Intracellular FL-CETP had a slightly lower molecular weight than secreted FL-CETP, likely due to incomplete carbohydrate side-chain processing. In these studies, the cellular content of FL-CETP protein typically exceeded that of ΔE9-CETP. However, since most FL-CETP is secreted, overall FL-CETP synthesis appears to greatly exceed that of ΔE9-CETP even though FL and ΔE9 CETP mRNA levels are similar.

Effect of CETP overexpression on cellular TAG

To assess whether CETP isoform expression alters cellular lipid metabolism, the extent of radiolabel incorporation into cellular lipids was measured following long-term labeling of cells with ^{3}H-oleate. Under such conditions, the extent of oleate incorporation approximates the mass of CE and TAG in cells. FL and E9-deleted CETP overexpression significantly increased ^{3}H-oleate incorporation into TAG and/or CE (Figure 1C). In cells overexpressing FL-CETP, TAG was increased by 75%, whereas the incorporation of ^{3}H-oleate into cellular TAG in ΔE9-CETP cells did not reach statistical significance ($P > 0.05$) by this 1-way ANOVA analysis. However, when directly compared only with null cells (t-test), TAG levels in cells expressing ΔE9-CETP were statistically increased more than 30% in each of two similar experiments. Cellular levels of the TAG precursor, diglyceride, were also markedly higher in FL and ΔE9 CETP expressing cells (8.5 ± 0.3 (null) versus 12.8 ± 0.8 (FL) and 12.0 ± 1.0 (ΔE9) cpm/μg protein, $P < 0.01$ vs null) (data not shown). FL or E9-deleted CETP overexpression increased cellular CE by ~30%. Changes in radiolabel incorporation into cellular PL were comparatively smaller and statistically significant only for cells expressing ΔE9-CETP. In contrast to cellular levels, overexpression of either FL or ΔE9 CETP impaired TAG secretion (Figure 1D). CE secretion was not reduced by overexpression of either CETP. Thus, both CETP isoforms appear to increase the cellular pools of TAG and CE and decrease the secretion of TAG into media. Since the amount of lipids secreted by cells is small compared to their cellular pools (note different y-axis scales of panels C versus D)

it is unlikely that the reduced secretion of TAG by CETP overexpressing cells significantly contributes to the higher intracellular content of these lipids.

To validate the findings from ^{3}H-oleate incorporation studies, the cellular mass of TAG was measured by direct enzymatic assay. Over expression of FL-CETP increased cellular TAG mass by ~25% (Figure 2A). The increase in TAG mass caused by overexpressing ΔE9-CETP did not quite reach significance (P = 0.0664). However, in cells grown in media containing 100 μM oleate to enhance TAG synthesis, the overexpression of either CETP isoform stimulated TAG accumulation (Figure 2A). To further characterize the nature of this increased TAG, cellular lipid droplets were stained with BODIPY 558/568 and imaged by fluorescence microscopy. Representative micrographs and quantitative characterization of lipid droplets are shown in Figures 2B–D. Compared to control cells transduced with null adenovirus, FL-CETP overexpression increased the number of lipid droplets per cell by approximately 50% and lipid droplet size by 40%. E9-deleted CETP overexpression did not significantly increase lipid droplet number (P = 0.096), but increase lipid droplet size almost two-fold.

Effect of CETP expression on lipid metabolism

The activities of several pathways involved in TAG homeostasis were directly assessed to determine their role in the increased TAG content of HepG2/C3A cell overexpressing CETP. The uptake of oleate, a TAG precursor (Jiang et al., 1998), by cells was not altered by CETP overexpression (Figure 3A). In addition, we also consider the possibility that the decreased turnover of TAG may cause the TAG accumulation in CETP overexpressed cells. HepG2/C3A cells were cultured in media containing ^{3}H-oleate to label TAG pool. Cells were then incubated in oleate-free media containing albumin and a fatty acyl-CoA synthetase inhibitor to prevent reincorporation of ^{3}H-oleate once cleaved from TAG. Although ^{3}H-TAG in CETP overexpressing cells was higher than control cells, the rate of TAG hydrolysis was similar among the three groups. (Figure 3B).

TAG synthesis was assessed from the incorporation of exogenous-supplied and endogenously-synthesized fatty acids during short-term studies. TAG synthesis from exogenous ^{3}H-oleate was significantly increased (Figure 3C). At 1 h, FL- and ΔE9-CETP overexpressing cells synthesized 18 and 24% more TAG, respectively, than null cells. These increases account for only a portion of the increased TAG observed above in CETP expressing cells. Thus, lipogenesis may also contribute to TAG accumulation. To assess this, cells were incubated for 4 h in media containing 30 μM ^{14}C-acetate to measure fatty acid and TAG lipogenesis. CETP overexpression increased the *de novo* synthesis of fatty acid from radiolabeled acetate up to 50% (Figure 3D). Incorporation of this newly synthesized fatty acid into TAG was increased 43% in cells overexpressing FL-CETP. In cells overexpressing ΔE9-CETP, TAG synthesis also trended higher but this increase was not statistical significance (P > 0.05) in this short-term experiment. We conclude that the elevated TAG content of cells overexpressing CETP is primarily due to increase biosynthesis. Elevated fatty acid biosynthesis may also contribute to the higher TAG in these cells.

Effect of CETP overexpression on genes involved in TAG synthesis

To investigate mechanism(s) underlying the increase in cellular TAG caused by CETP overexpression, gene expression of key enzymes involved in TAG synthesis were investigated. Unexpectedly, the only effect of FL and E9-deleted CETP overexpression was to suppress the expression of *DGAT1* and *DGAT2* (Table 1). Regardless of the presence of oleate, CETP overexpression suppressed both *DGAT1* and *DGAT2* mRNA levels by more than 50%. Collectively, these results indicate that neither FL- nor ΔE9-CETP induces TAG accumulation through increased mRNA levels of these key genes.

Effect of CETP knockdown on lipid metabolism in HepG2/C3A cells

To validate these overexpression studies, the effect of depleting CETP on lipid metabolism was investigated. Using a siRNA approach, CETP mRNA levels were reduced by 60% (Figure 4A) and the amount of CETP secreted into the media was 50% of control (Figure 4B). Although not impacting the TAG content of cells gown without oleate addition, in CETP knockdown cells cultured in media containing 100 uM oleate, cellular TAG mass was decreased 24% (Figure 4C). A similar reduction in cellular TAG was also seen in long-term ^{3}H-oleate incorporation studies (Figure 4D). CETP knockdown also increased the amount of TAG secreted into the media by ~20% (Figure 4E). PL radioactivity in cells or in the media was not altered by CETP knockdown. The impact of CETP knockdown on lipogenesis was measured in ^{14}C-acetate experiments as described above. siCETP cells synthesized 30% less fatty acid and 23% less ^{14}C-labeled TAG than siRNA control cells (Figure 4F). Collectively, even though CETP expression in control HepG2/C3A cells is low, inhibition of CETP synthesis produces a phenotype that is the opposite of that observed in cells overexpressing either CETP isoform. That is, cellular TAG mass and the *de novo* synthesis of fatty acids and their incorporation into TAG are reduced, whereas the secretion of TAG into the media is increased.

Effect of FL-CETP overexpression on Caco-2 cells

The foregoing data show that overexpression of either FL- or ΔE9-CETP increases cellular TAG levels in HepG2/C3A cells. For ΔE9-CETP, this reproduces the phenotype we previously observed in SW872 adipocytes (Izem et al., 2020). However, the findings here for FL-CETP are contrary to those observed in SW872 cells where FL-CETP overexpression decreased cellular TAG (Izem et al., 2015). This surprising finding suggests that the effect of FL-CETP on cellular lipid metabolism may differ between cell types. To investigate this further, we examined the effect of FL-CETP overexpression on lipid metabolism in Caco-2 cells - an intestinal epithelial cell. Like hepatocytes, a major function of intestinal epithelial cells is to take up lipids, and to repackage and secrete them in lipoproteins.

Caco-2 cells naturally express CETP, and the amount of CETP synthesized is regulated by lipids (Dessi et al., 1997; Izem & Morton, 2001). To examine the impact of CETP overexpression, Caco-2 cells were transfected with pCDNA3 containing FL-CETP sequence. This increased the synthesis of FL-CETP about 4-fold based on CETP activity contained in conditioned media. Cells were subsequently incubated long-term with ^{3}H-oleate/BSA to label cellular lipid pools. Like that seen with HepG2/C3A cells, FL-CETP overexpression in Caco-2 cells increased cellular levels of TAG (Figure 5A). Although

not statistically increased in this multiple comparison analysis ($P > 0.05$), when compared directly with control Caco-2 cells, data from 3 similar experiments show that TAG levels in cells overexpressing FL-CETP were 188 ± 33 % of control ($P < 0.01$). Unlike HepG2/C3A cells, however, FL-CETP overexpression stimulated TAG secretion into the media almost 2-fold (Figure 5B). FL-CETP expression had no effect on cellular (Figure 5C) or secreted (Figure 5D) phospholipid.

The effect of FL-CETP expression on CE, another lipid transferred by CETP, was similar to that of TAG. Among 3 similar experiments, FL-CETP overexpression increased cellular CE by 1.5-fold compared to 1.8-fold for TAG, and the secretion of CE was increased 1.9-fold compared to 2.4-fold for TAG (data not shown).

To further characterize the elevated TAG secreted by Caco-2 cells overexpressing FL-CETP, cells were treated with CI-1011. Compound CI-1011 inhibits apolipoprotein B (ApoB) lipidation, causing ApoB to be degraded intracellularly and preventing the secretion of TAG (Wilcox et al., 1999; Taghibiglou et al., 2020). CI-1011 had no statistically significant impact on the cellular content of TAG (Figure 5A) or PL (Figure 5C) compared to the same cell not treated with CI-1011 ($P > 0.05$). However, in the presence of CI-1011, cells overexpressing FL-CETP contained almost 2-fold more TAG than control cells. The amount of TAG secreted into the media by control and FL-CETP overexpressing Caco-2 cells was reduced by more than 70% and the higher secretion of TAG by cells overexpressing FL-CETP was completely blocked (Figure 5B). CI-1011 had minimal effect on PL secretion (Figure 5D). These data show that the TAG secreted into the media by both control and FL-CETP overexpressing Caco-2 cells is contained in ApoB-containing lipoproteins.

DISCUSSION

Secreted FL-CETP has a well-characterized role in the metabolism of circulating plasma lipoproteins (Klerkx et al., 2006; Oliveira, 2011). ΔE9-CETP, which remains intracellular, may contribute to lipoprotein metabolism by inhibiting the biosynthesis of FL-CETP (Quinet et al., 1993; Izem et al., 2020). In addition to these functions, we have shown that these CETP isoforms also influence cellular lipid homeostasis. When expressed separately in SW872 cells, an adipose-derived cell line, ΔE9-CETP overexpression increases cellular TAG levels, whereas FL-CETP overexpression has the opposite effect (Izem et al., 2015; Izem et al., 2020). These changes in TAG storage are the result of isoform-specific alterations in the rates of TAG synthesis and turnover. However, multiple studies (Blake et al., 1994; Zhou et al., 2006; Salerno et al., 2007; Terán-García et al., 2008; Salerno et al., 2009; Lucero et al., 2011; Liaw et al., 2014; Fadaei et al., 2018; Morton et al., 2021) suggest that lipid levels in adipose and liver may respond differently to changes in FL-CETP expression. Here, we investigated this possibility in HepG2/C3A hepatocytes. As observed in SW872 adipocytes, ΔE9-CETP overexpression in HepG2/C3A hepatocytes significantly increased cellular TAG levels. FL-CETP overexpression in hepatocytes also induced TAG accumulation, which is the opposite of that observed in SW872 cells. This novel response to FL-CETP overexpression by HepG2/C3A cells was reproduced in Caco-2 cells.

The mechanisms by which CETP impacts cellular TAG storage are still being resolved. Both CETP isoforms promote CE and TAG transfer from isolated microsomes to lipid droplets in vitro (Izem et al., 2020), suggesting a role in intracellular lipid movement. Consistent with this, in CETP-deficient cells, newly synthesized CE and TAG are ineffectively moved from their site of synthesis in the endoplasmic reticulum (ER) to lipid droplets (Izem & Morton, 2007). In both SW872 adipocytes (Izem & Morton, 2007) and in HepG2/C3A cells (reported here), this inhibition of CETP expression decreased cellular TAG levels. Therefore, one function of FL and ΔE9 CETP inside cells may be to facilitate lipid movement between organelles to promote storage.

Other mechanisms may contribute to the TAG storage phenotype observed here. In FL-CETP overexpressing cells, a subfraction of CETP co-localizes with lipid droplets concomitant with a change in the lipid droplet proteome, including altered perilipins 1, 2, and 3 content (Izem et al., 2015). Thus, CETP may directly influence TAG metabolism by altering the binding of metabolically important proteins to the lipid droplet surface. For example, the increased TAG content of SW872 cells expressing ΔE9-CETP (Izem et al., 2020) and of HepG2/C3A cells expressing either CETP isoform (shown here) is due, in part, to increased TAG synthesis. One of the rate limiting enzymes in TAG synthesis in lipogenic tissues, diacylglycerol acyltransferase-2, binds to the lipid droplet surface to facilitate TAG assembly (Kuerschner et al., 2008; McFie et al., 2011; McFie et al., 2018). Also, in SW872 cells overexpressing FL-CETP, their decreased TAG content correlates with increased TAG hydrolysis (Izem et al., 2015), which is initiated by lipid droplet-associated adipose triglyceride lipase. Since CETP is capable of binding to diverse membranes (Morton, 1990), it is plausible that FL-CETP, and perhaps ΔE9-CETP, may bind to the lipid droplet surface and modulate lipid metabolism by altering the binding of diacylglycerol acyltransferase-2, adipose triglyceride lipase, or other proteins directly involved in lipid homeostasis. Because the proteome of lipid droplets varies significantly between different tissues (Beilstein et al., 2013; Su et al., 2014; Bersuker & Olzmann, 2017; Zhang & Liu, 2019), it would not be unexpected if CETP binding to lipid droplets has different consequences between cells types such as adipocytes and hepatocytes. In addition to altering the lipid droplet surface, CETP may also directly promote TAG synthesis by facilitating the transfer of diacylglycerol, a recently identified CETP substrate (Greene et al., 2015), to lipid droplets where diacylglycerol acyltransferase-2 resides.

FL-CETP expression has different effects on TAG accumulation in SW872 adipocytes and HepG2/C3A cells. This may be due to a fundamental difference in the handling of lipids by adipocytes versus hepatocytes. Adipocytes store TAG, and then hydrolyze it and release fatty acids to the extracellular compartment when needed. On the other hand, lipid droplet TAG in hepatocytes is routed to the ER for TAG-rich lipoprotein assembly before secretion. This difference provides additional metabolic steps where CETP, especially ER luminal CETP, could impact TAG homeostasis and elicit a unique TAG storage phenotype. In this context, it is interesting that FL-CETP expression in Caco-2 cells, which also export their stored TAG in the form of TAG-rich lipoproteins, produces the same elevated cellular TAG phenotype as it does in HepG2/C3A cells.

TAG is primarily secreted by cells in ApoB-containing lipoproteins (Dashti & Wolfbauer, 1987). We were intrigued by the finding that overexpression of CETP in HepG2/C3A and Caco-2 cells has the same effect on cellular TAG levels but opposite effects on TAG secretion. In general, the increased cellular TAG pool in HepG2/C3A and Caco-2 cells overexpressing FL- or ΔE9-CETP would be expected to drive the secretion of ApoB lipoproteins containing higher TAG content per particle (Dashti et al., 1990). This likely explains the greater TAG secretion by Caco-2 cells overexpressing CETP but provides no insight into the reduced TAG secretion by HepG2/C3A cells overexpressing CETP. It is possible that the difference between HepG2/C3A and Caco-2 cell TAG secretion arises from experimental differences in the mode (adenovirus versus plasmid) or the extent of CETP overexpression in these two cell types. However, in our previous CETP expression studies with SW872 adipocyte cells, the general phenotype produced by CETP overexpression was not dependent on the expression vector or the extent of overexpression (Izem et al., 2015; Izem et al., 2020). And in studies not shown here, we observed that transformation of HepG2/C3A cells with less adenovirus (5 instead of 30 pfu/cell) still caused these cells to accumulate more TAG and secrete less TAG than control cells. Alternatively, the reduced TAG secretion by HepG2/C3A but not Caco-2 cells may relate to differences in TAG-rich lipoprotein biosynthesis in these cells. HepG2/C3A cells secrete TAG in ApoB100-containing particles, whereas Caco-2 cells secrete TAG in both ApoB100 and ApoB48-containing particles. (Nicodeme et al., 1999). ApoB100 synthesis and secretion requires co-translational lipidation by microsomal lipid transfer protein (MTP) (Sirwi et al., 2018). ApoB48 synthesis is less dependent on these lipidation events (Van Greevenbroek et al., 1998). Further, based on MTP inhibition studies, ApoB100 secretion by HepG2 cells is more sensitive to reduced MTP activity than is ApoB100 secretion by Caco-2 cells (Jamil et al., 1996; Van Greevenbroek et al., 1998). Therefore, if increased levels of CETP in the ER lumen interfere with the ApoB lipidation process, then the secretion of TAG by CETP-overexpressing HepG2/C3A cells will be the most sensitive to this disruption. Whether CETP can directly augment or interfere with the ApoB lipidation process is not known. At least in vitro, FL-CETP can redistribute lipids between smooth and rough endoplasmic reticulum (Hashimoto et al., 1984), which may alter TAG availability for lipoprotein assembly.

In summary, the data presented here confirm and extend our understanding of the roles of full length and exon-9 deleted forms of CETP in cellular lipid metabolism. The data show that FL-CETP impacts cellular lipid homeostasis differently in cells whose primary function is to store lipid (adipocytes) versus those involved in lipoprotein assembly and secretion (hepatocytes, intestinal epithelial cells). Unlike adipocytes, in hepatocytes overexpressing either FL-CETP and ΔE9-CETP, or Caco-2 cells overexpressing FL-CETP, CETP promotes the accumulation of cellular TAG. These findings provide a possible mechanism for the direct association between CETP expression and hepatic steatosis observed in human studies (Lucero et al., 2011; Adams et al., 2012; Aller et al., 2018; Fadaei et al., 2018; Yoo et al., 2019).

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Abbreviations:

ACACA	acetyl-CoA carboxylase alpha
ApoB	apolipoprotein B
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
ΔE9	exon 9-deleted
DGAT1	diacylglycerol O-acyltransferase 1
DGAT2	diacylglycerol O-acyltransferase 2
ER	endoplasmic reticulum
FASN	fatty acid synthase
FL	full-length
GPAM	glycerol-3-phosphate acyltransferase, mitochondrial
MTP	microsomal transfer protein
PL	phospholipid
TAG	triacylglycerol

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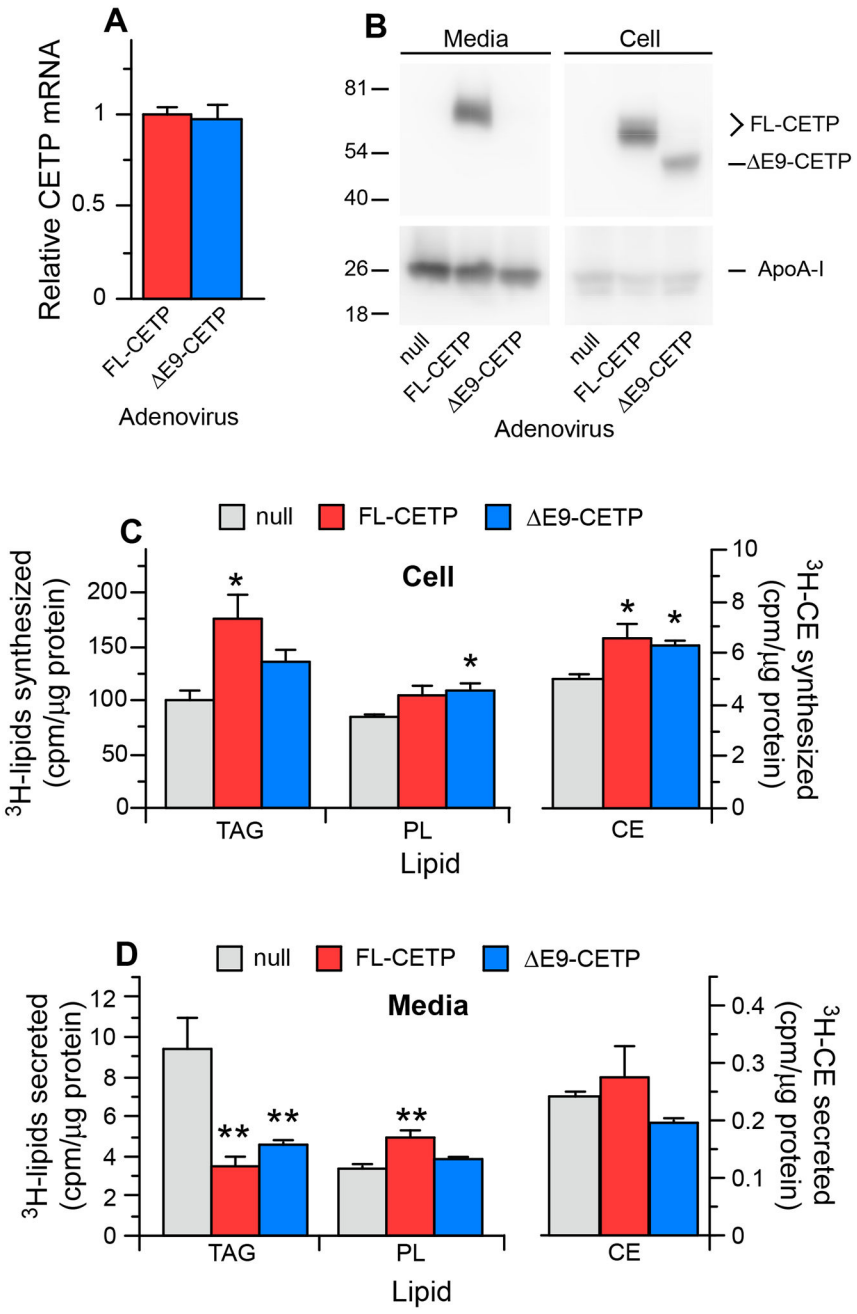

Figure 1.

Expression of CETP in HepG2/C3A cells. Cells were transduced with null, FL-CETP or ΔE9-CETP adenoviruses as described in Methods. Panel A - CETP mRNA levels determined by qPCR. (n = 4). Panel B - Immunoblot of media and cell homogenates for CETP (top). An immunoblot for ApoA-1 (bottom) is shown as a loading control. Panels C (Cell) and D (Media) - Cells overexpressing FL- or ΔE9-CETP were incubated in 100 μM ^{3}H-oleate/BSA for 48 h. ^{3}H incorporated into cholesteryl ester (CE), triacylglycerol (TAG), and phospholipid (PL) were quantified by scintillation counting. (n = 4). *P < 0.05 vs null, **P < 0.01 vs null.

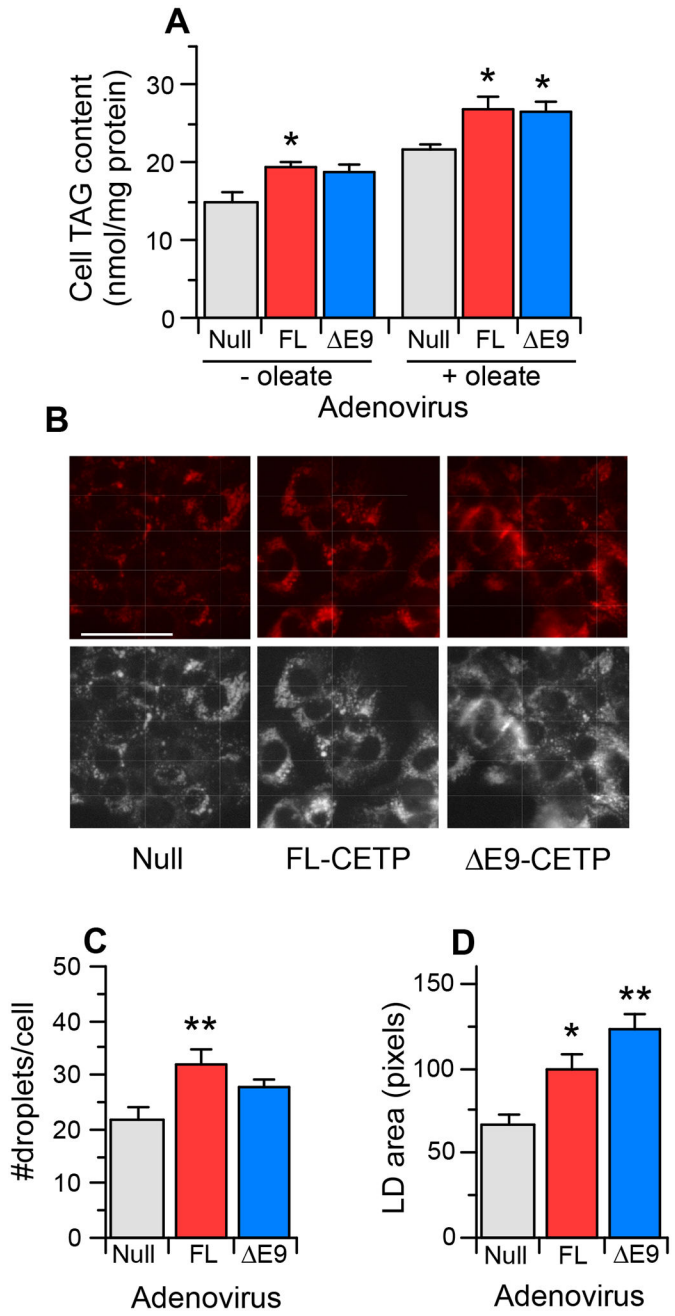

Figure 2.

TAG accumulation in HepG2/C3A cells overexpressing FL or ΔE9-CETP. Panel A - Cells were incubated without or with 100 μM oleate/BSA for 48 h. Intracellular TAG levels were quantified enzymatically. (n = 4). Panel B – Representative fluorescence micrographs of control and CETP transduced cells. Cells were incubated with 100 μM oleate/BSA for 24 h to promote the development of lipid droplets. Cells were fixed, and lipid droplets stained with BODIPY 558/568. Bar = 50 μm. Panels C and D – quantitation of lipid droplet number and size (n = 8). Lipid droplets in eight fields containing 131–150 cells/field were quantified. Abbreviations: null, null adenovirus; FL, FL-CETP adenovirus; ΔE9, ΔE9-CETP

adenovirus. *P < 0.05 vs null with the same oleate treatment, **P < 0.01 vs null with the same oleate treatment.

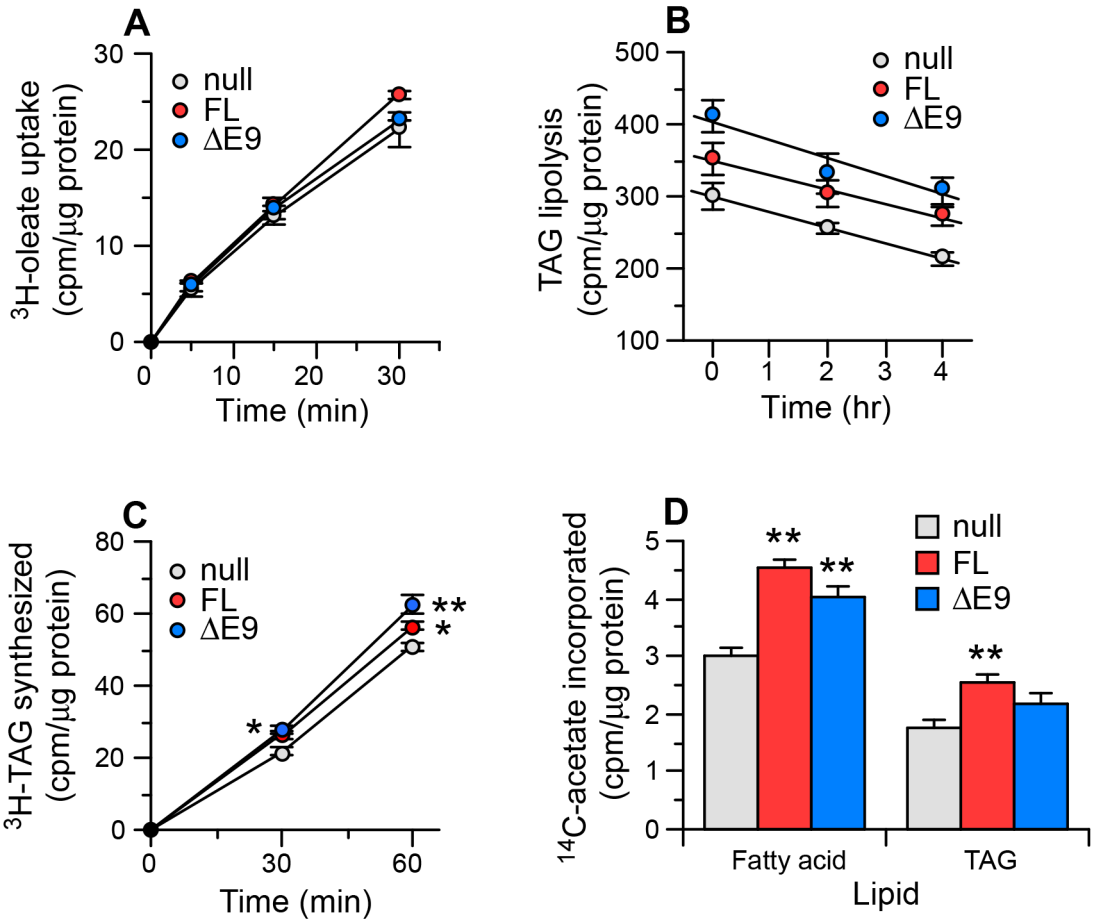

Figure 3.

Lipid metabolism in HepG2/C3A cells overexpressing FL- or ΔE9-CETP. Panel A - FL and ΔE9 overexpressed cells were incubated with 100 μM ^{3}H-oleate/BSA for the indicated time, and then the cellular content of ^{3}H was determined. (n = 2). Panel B – FL- and ΔE9-CETP overexpressing cells were incubated in 100 μM ^{3}H-oleate/BSA for 24 h. After washing in oleate-free media, cells were incubated in media containing 10 μM Triacsin C for the indicated time. Lipids were extracted, fractionated by thin layer chromatography, and ^{3}H-TAG quantified by scintillation counting. (n = 4). Panel C - To measure the TAG synthetic rate, FL and ΔE9 overexpressed cells were incubated with 100 μM ^{3}H-oleate/BSA for the indicated times. Lipids were extracted, fractionated by thin layer chromatography, and ^{3}H-TAG quantified by scintillation counting. (n = 3). Panel D – Cells were incubated in 30 μM ^{14}C-acetate for 4 h to determine the *de novo* synthesis of fatty acid (FFA) and TAG. Lipids were extracted from cells, fractionated by thin layer chromatography, and ^{14}C quantified by scintillation counting. (n = 4). Abbreviations: null, null adenovirus; FL, FL-CETP adenovirus; ΔE9, ΔE9-CETP adenovirus. *P < 0.05 vs null, **P < 0.01 vs null.

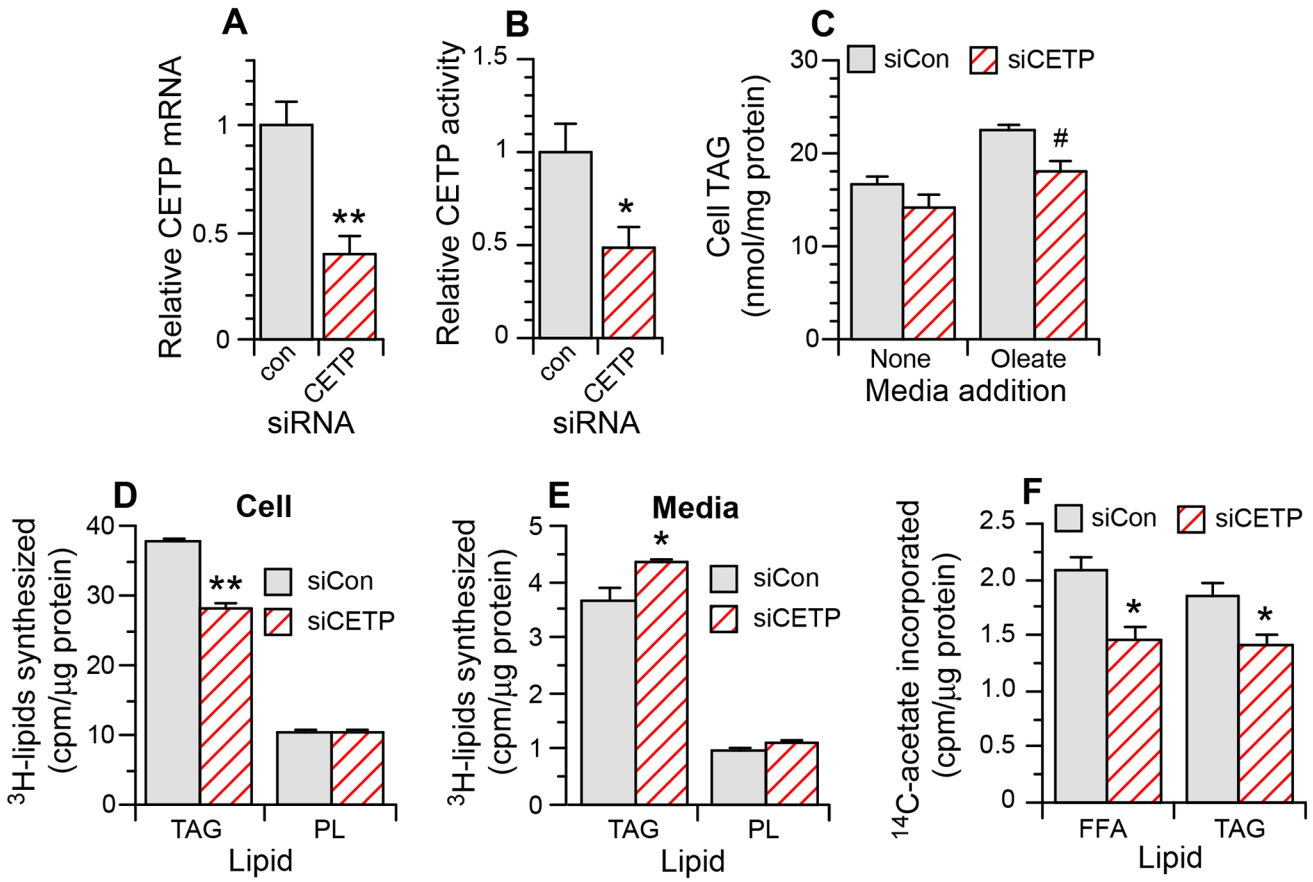

Figure 4.

Effect of CETP knockdown on lipid metabolism in HepG2/C3A cells. Panel A – CETP mRNA levels in cells ± CETP siRNA were determined by qPCR. (n = 4). Panel B – CETP present in 48 h-conditioned media was determined by a CETP activity assay. (n = 5). Panel C- Cell TAG mass was quantified by an enzymatic assay following incubation of cells with 100 µM oleate/BSA for 48 h. (n = 6). Panels D and E – ^{3}H oleate incorporated into cell (Panel D) and media (panel E) lipid was measured following incubation with 100 µM ^{3}H-oleate/BSA for 48 h. Panel F – To quantify the *de novo* synthesis of free fatty acids (FFA) and TAG, cells were incubated in 30 µM ^{14}C-acetate for 4 h. Lipids were extracted from cells, fractionated by thin layer chromatography, and ^{14}C quantified by scintillation counting. (n = 4) Abbreviations: siCon, control siRNA; siCETP, CETP siRNA. *P < 0.05 vs siCon, **P < 0.01 vs siCon. Panel C - $^{\#}$P < 0.05 vs siCon + oleate.

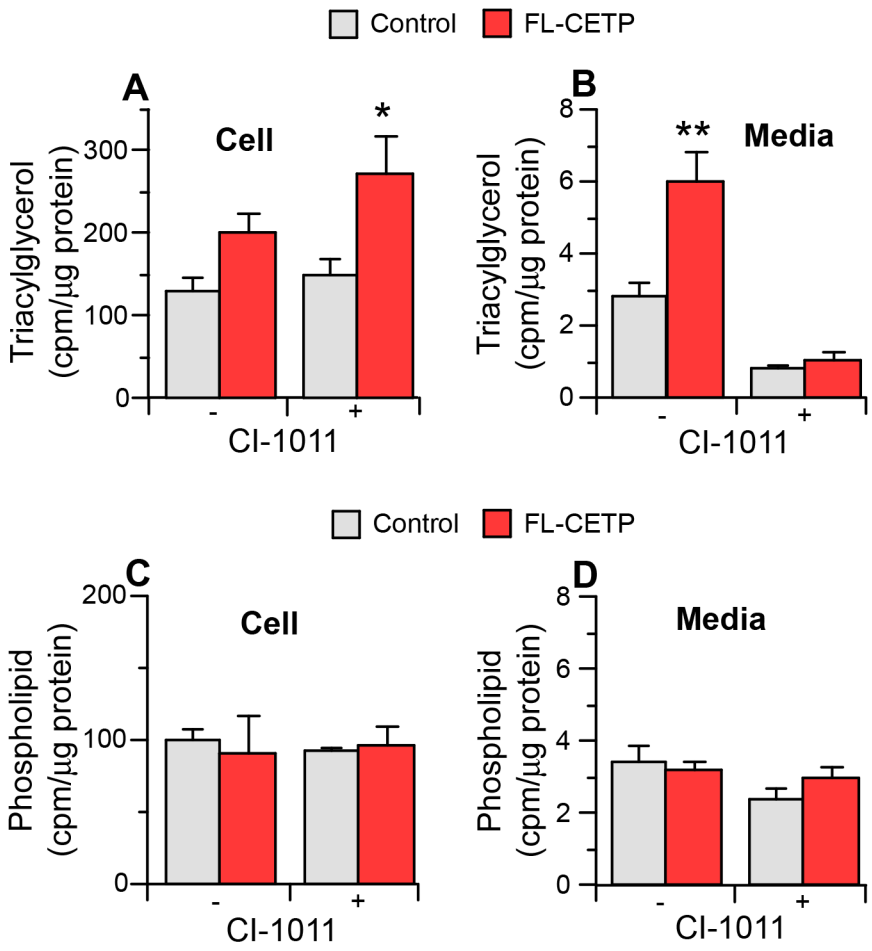

Figure 5.

Effect of transient FL-CETP expression on cellular lipids in Caco-2 cells. Cells were transfected with 10 µg control or FL-CETP plasmid. After 18 h, cells were washed and incubated for 24 h in media containing 100 µM ^{3}H-oleate/BSA ± 10 µM CI-1011. CI-1011 blocks ApoB secretion. Cells (panels A and C) and media (panels B and D) were collected and their content of radiolabeled TAG and phospholipid (PL) was determined as described in the Methods. Values are the mean ± SD, n = 3. *P < 0.05 vs control with same CI-1011 treatment, **P < 0.01 vs control with same CI-1011 treatment.

Table 1

Relative expression of lipid synthesis genes.[a]

		Relative mRNA levels				
Oleate	**Adenovirus**	***ACACA***	***FASN***	***GPAM***	***DGAT1***	***DGAT2***
−	Null	1.00±0.10	1.00±0.24	1.00±0.13	1.00±0.07	1.00±0.03
−	FL-CETP	0.55±0.01	0.46±0.03	1.11±0.15	0.35±0.03[c]	0.50±0.03[c]
−	ΔE9-CETP	0.52±0.01	0.44±0.01	0.27±0.04	0.39±0.07[c]	0.50±0.04[c]
+	Null	0.78±0.09	0.84±0.17	1.15±0.15	0.98±0.04	1.15±0.04
+	FL-CETP	0.88±0.27	0.60±0.13	0.73±0.12	0.32±0.07[c]	0.50±0.03[c]
+	ΔE9-CETP	0.45±0.05	0.40±0.01	0.46±0.10	0.51±0.03[b]	0.68±0.05[b]

[a]Cells were transduced with the indicated adenovirus. After overnight incubation in serum containing media, cells were washed and incubated in serum-free media ± 100 μM oleate/BSA for 24 h. mRNA levels were quantified by qPCR and normalized to GAPDH mRNA. Abbreviations: ACACA, acetyl-CoA carboxylase alpha; FASN, fatty acid synthase; GPAM, glycerol-3-phosphate acyltransferase, mitochondrial; DGAT1, diacylglycerol O-acyltransferase 1; DGAT2, diacylglycerol O-acyltransferase 2.

Values are the mean ± SE (n = 4).

[b]$P < 0.05$ vs Null with the same oleate treatment.

[c]$P < 0.01$ vs Null with the same oleate treatment.