

# Overexpression of full-length cholesteryl ester transfer protein in SW872 cells reduces lipid accumulation

Lahoucine Izem,\* Diane J. Greene,\* Katarzyna Bialkowska,<sup>†</sup> and Richard E. Morton<sup>1,\*</sup>

Department of Cellular and Molecular Medicine,\* and Department of Molecular Cardiology,<sup>†</sup>  
Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195

**Abstract** Cells produce two cholesteryl ester transfer protein (CETP) isoforms, full-length and a shorter variant produced by alternative splicing. Blocking synthesis of both isoforms disrupts lipid metabolism and storage. To further define the role of CETP in cellular lipid metabolism, we stably overexpressed full-length CETP in SW872 cells. These CETP<sup>+</sup> cells had several-fold higher intracellular CETP and accumulated 50% less TG due to a 26% decrease in TG synthesis and 2.5-fold higher TG turnover rate. Reduced TG synthesis was due to decreased fatty acid uptake and impaired conversion of diglyceride to TG even though diacylglycerol acyltransferase activity was normal. Sterol-regulatory element binding protein 1 mRNA levels were normal, and although *PPAR $\gamma$*  expression was reduced, the expression of several of its target genes including adipocyte triglyceride lipase, *FASN*, and *APOE* was normal. CETP<sup>+</sup> cells contained smaller lipid droplets, consistent with their higher levels of perilipin protein family (PLIN) 3 compared with PLIN1 and PLIN2. Intracellular CETP was mostly associated with the endoplasmic reticulum, although CETP near lipid droplets poorly colocalized with this membrane. A small pool of CETP resided in the cytoplasm, and a subfraction coisolated with lipid droplets. These data show that overexpression of full-length CETP disrupts lipid homeostasis resulting in the formation of smaller, more metabolically active lipid droplets.—Izem, L., D. J. Greene, K. Bialkowska, and R. E. Morton. Overexpression of full-length cholesteryl ester transfer protein in SW872 cells reduces lipid accumulation. *J. Lipid Res.* 2015. 56: 515–525.

**Supplementary key words** lipid metabolism • lipid turnover • lipid droplets • cellular localization • gene expression

In cells, multiple proteins transport phospholipids, sterols, and fatty acids between organelles (1, 2). However, little is known about the molecular mechanisms of cholesteryl ester (CE) and TG transport. A candidate protein for this function is cholesteryl ester transfer protein (CETP). CETP's role in CE and TG transport in plasma and its impact on lipoprotein metabolism are well known (3–5).

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CETP is also present inside cells where two isoforms exist: full-length CETP, which is equivalent to plasma CETP, and exon 9-deleted CETP, a smaller form derived from alternatively spliced mRNA (6). The function of exon 9-deleted CETP is poorly understood. Overexpression studies suggest that exon 9-deleted CETP may bind to full-length CETP and hinder its secretion (6, 7), although this role was not supported by subsequent transgenic mouse studies (8).

Multiple studies suggest that intracellular CETP modulates lipid metabolism. Cell-associated CETP promotes CE uptake from HDL, stimulates the cell's ability to efflux cholesterol, and influences the storage of CE in cells (9–12). Acute suppression of CETP biosynthesis by antisense oligonucleotides in SW872 cells reduces cholesterol synthesis and increases CE accumulation (13). SW872 cells chronically deficient in CETP manifest more profound alterations in lipid metabolism including reduced capacity to store TG (14). A role for CETP in cellular lipid storage is further supported by observations in transgenic mice. Adipose tissue-specific expression of human CETP in mice results in smaller adipocytes containing less TG and cholesterol and significantly reduces the expression of key lipogenic genes (15). In hypertriglyceridemic mice, CETP expression normalizes subcutaneous adipose depots and visceral adipocyte size (16). And in humans, a CETP gene variant that affects the coding sequence of both full-length and exon 9-deleted CETP is associated with increased adiposity following long-term overfeeding (17).

Our initial studies in intracellular CETP, which used a molecular approach that reduced both full-length and exon 9-deleted CETP expression, identified multiple aberrations in lipid metabolism and storage (13, 14). In that

Abbreviations: CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; CETP<sup>+</sup> cells, cells overexpressing full-length CETP; DG, diglyceride; DGAT, diacylglycerol acyltransferase; *FABP4*, fatty acid binding protein 4; *HMGCR*, HMG-CoA reductase; *LPIN1*, phosphatidate phosphatase; PLIN, perilipin protein family; *PNPLA2*, adipocyte triglyceride lipase; qPCR, real-time quantitative PCR; *SCL2A4*, glucose transporter type 4 (GLUT4); *SREBF1*, sterol regulatory element-binding protein 1.

To whom correspondence should be addressed.  
e-mail: mortonr@ccf.org

study, we developed an *in vitro* assay that measured the ability of cytosolic CETP to promote the transfer of CE and TG from an endoplasmic reticulum-enriched fraction to lipid droplets. We observed that cytosol from control cells promoted lipid transfer between these isolated organelles, whereas cytosol from CETP-deficient cells was ineffective in this activity. We recently determined that the interorganelle lipid transfer activity in control cytosol could be mediated by full-length CETP alone. This finding prompted us to investigate how overexpression of full-length CETP in SW872 cells affects their lipid metabolism.

We report here that an increase in intracellular full-length CETP levels also induces marked changes in lipid homeostasis, including a 2-fold reduction in TG storage due to reduced TG synthesis and enhanced turnover. Increased CETP synthesis also altered the expression of multiple genes involved in lipid metabolism and reduced the CE content of these cells despite an increase in its synthetic rate. Cellular localization studies show that a subfraction of CETP is in the cytoplasm and a portion of this pool coisolates with lipid droplets, further supporting a role for CETP in the formation of these dynamic structures.

## EXPERIMENTAL PROCEDURES

### Materials

[9,10(n)-<sup>3</sup>H]oleic acid was from Perkin-Elmer Life Sciences (Boston, MA). Stock 2.7 mM <sup>3</sup>H-oleate/BSA (~2,500 cpm/nmol oleate) and unlabeled oleate/BSA (7:1 mol ratio of oleate to BSA) were prepared as previously described (18). HDL was prepared from human plasma by sequential ultracentrifugation (19), dialyzed extensively versus 0.9% NaCl, 0.02% EDTA, then sterile filtered. 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).

### Preparation of cells overexpressing CETP

The human liposarcoma cell line, SW872, was purchased from American Type Culture Collection (Manassas, VA). Cells were grown in DMEM/Ham's F-12 (1:1) containing 10% FBS (Atlas Biologicals, Fort Collins, CO) and 50 µg/ml penicillin/streptomycin in 5% CO<sub>2</sub>/95% air at 37°. Unless stated otherwise, cells were ~90% confluent at the start of an experiment, at which time cells were further incubated as described below.

Full-length human CETP including the natural Kozak sequence and the stop codon was amplified by PCR from a cDNA construct (pCETP.11) purchased from American Type Culture Collection. The fragment (position -46 to +1,542, NM\_000078) was inserted into pCDNA3 using *Bam*HI and *Xba*I restriction sites. SW872 cells at 70% confluence were transfected with native pCDNA3 or pCDNA3-CETP plasmid using Lipofectamine 2000 transfection reagent following the manufacturer's directions (Life Technologies, Grand Island, NY). Transfectants were selected using 500 µg/ml G418 (Life Technologies). After 10–15 days, clones were picked and subcultured under the same selection conditions. Both vector-transfected SW872 cells and native SW872 cells were used as control as indicated in the text and fig-

ure legends. No significant differences were noted between these cells for the end points reported here.

Clones were tested for CETP expression by measuring CETP activity in 48 h conditioned medium collected in the absence of serum (3, 13). Volumes of conditioned media assayed were normalized by the cell count per well. Assay aliquots typically reflected the amount of media conditioned by ~6 × 10<sup>5</sup> cells. Normalized aliquots of conditioned media were also concentrated 8-fold by cold acetone precipitation, then applied to 4–20% SDS-PAGE gels (Lonza, Rockland, ME). Cells were lysed by passage through a 26-gauge needle, centrifuged at 1,000 *g* for 10 min, and the supernatant (25 µg protein) fractionated by electrophoresis. After transfer to polyvinylidene difluoride membranes, CETP was detected by TP2 antibody (13). Clones overexpressing CETP were designated as CETP<sup>+</sup> cells. Trypsinized CETP<sup>+</sup> cells adhered less efficiently when passaged but had the same growth rate as vector-transfected control cells after 2 days in culture.

CETP-deficient SW872 cells, prepared as previously described (14), were used in select experiments.

### Oleate incorporation into TG and its precursors

Cells were washed with PBS and incubated in OptiMEM (Life Technologies) for 24 h before the addition of prewarmed 200 µM <sup>3</sup>H-oleate/BSA in OptiMEM. At the indicated time, the media was removed and ice cold PBS was added to cells. Culture plates were kept on ice until cells were scraped. A zero time blank was used to correct for any metabolism that occurred during sample processing. Cellular lipids were extracted (20) and separated by thin layer chromatography. Initially, plates were developed halfway in a solvent system containing chloroform-acetone-methanol-acetic acid-water (60:80:20:20:10, v/v). After drying, chromatography continued in a second system of hexanes-diethyl ether-acetic acid (80:20:1, v/v). 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.

### TG and CE synthetic rates

Cells were cultured in growth media containing 200 µM unlabeled oleate/BSA for 48–72 h to initiate droplet formation. Cells were then washed with media and incubated for 0–4 h in the same media containing 200 µM <sup>3</sup>H-oleate/BSA. Synthesis was stopped by removing the media and washing cells with cold PBS. Cells were kept on ice until released by trypsin. Cellular lipids were extracted (21) and separated by thin layer chromatography (hexanes-diethyl ether, 70:30, v/v). TG and CE bands were scraped and <sup>3</sup>H quantified by scintillation counting.

To measure the transfer of newly synthesized TG to lipid droplets, cells were incubated as described above, washed, and then suspended in 500 µl cold hypotonic lysis buffer [10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM sodium fluoride, 200 mM sucrose, and EDTA-free protease inhibitor cocktail (Roche Applied Science (Indianapolis, IN))] containing 300 µM diethylumberylferyl phosphate (Sigma-Aldrich Corp.). After 20 min, cells were homogenized by 10 strokes with a motor-driven Kontes pellet pestle (Sigma-Aldrich Corp.). Cell homogenates were centrifuged at 2,000 *g* for 10 min, and the resulting supernatant was centrifuged at 100,000 *g* for 1 h to yield a lipid droplet-rich fraction at the top and endoplasmic reticulum-enriched fraction at the bottom of the tube. Lipids in these fractions were extracted (21) and separated by thin layer chromatography as above.

### TG hydrolysis

To determine the rate of TG turnover, cells were incubated in growth media supplemented with 200 µM <sup>3</sup>H-oleate/BSA for

24 h to label the cellular TG pool. The cells were washed with warm media, then either harvested ( $t = 0$ ) or incubated for the indicated times in media containing 100  $\mu\text{g/ml}$  HDL and 0.1% BSA in the presence of 10  $\mu\text{M}$  triacsin C (Sigma-Aldrich Corp.), a fatty acyl-CoA synthetase inhibitor. Cells were harvested, and lipids extracted (21) and fractionated by thin layer chromatography as described above.

### Fatty acid uptake

Cells were incubated in media containing 200  $\mu\text{M}$   $^3\text{H}$ -oleate/BSA for the indicated time. At each time point, the cells were washed with cold PBS, released from the dish by trypsin, washed three additional times with cold PBS, and solubilized in 0.1 N NaOH. The  $^3\text{H}$ -content was determined by scintillation counting.

### Lipid droplet staining

Cells were cultured on glass cover slips in 12-well plates. At 70% confluence, 100  $\mu\text{M}$  oleate/BSA was added, and the cells were incubated for 24 h. Cells were fixed using 4% formaldehyde, then stained for 15 min with 200 ng/ml BODIPY 558/568  $\text{C}_{12}$  in PBS. The coverslips were applied using 4',6-diamidino-2-phenylindole (DAPI)-containing mounting solution. Cells were visualized by confocal microscopy, and lipid droplet size and number determined by ImagePro software (Media Cybernetics, Silver Spring, MD).

### Cellular localization of CETP

CETP<sup>+</sup> cells were incubated in growth media containing 100  $\mu\text{M}$  oleate/BSA to induce lipid droplet formation. Lipid-loaded cells were fixed for 30 min with 4% formaldehyde, permeabilized for 30 min with 0.01% digitonin (EMD Chemical Inc., San Diego, CA), and then blocked with PBS containing 5% horse serum for 1 h at room temperature. Subsequently, cells were reacted with mouse anti-human CETP antibody (TP2, 1/200) and rabbit anti-human calnexin (1/200) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 24 h.

For CETP detection, antigen-antibody complexes were reacted with biotin-conjugated goat anti-mouse IgG (1/1,000) for 1 h and then with Alexa Fluor 488 streptavidin (1/1,000) for 15 min. For calnexin detection, cells were incubated with Alexa Fluor 647 chicken-anti rabbit IgG (1/1,000) for 1 h. After immunostaining, the cells were incubated with PBS containing 200 ng/ml of BODIPY 558/568  $\text{C}_{12}$  for 15 min to stain lipid droplets. All fluorescent reagents were from Life Technologies. The cell were washed three times with PBS, then mounted on microscope slide using DAPI-containing mounting solution. Confocal images were obtained using a Leica TCS-NT confocal laser-scanning microscope.

The cellular distribution of CETP was also determined by Western blot of cell fractions. Lipid-loaded cells were lysed in hypotonic media as described above. The homogenate was centrifuged for 10 min at 2,000  $g$  at 4°C. Supernatant aliquots containing equal protein (22) were centrifuged at 100,000  $g$  for 1 h to yield lipid droplet (top), cytosolic (remaining supernatant), and endoplasmic reticulum-containing (pellet) fractions. Fractions were adjusted to the same volume using lysis buffer, and equal volumes were loaded on 4–20% SDS-PAGE gels. Western blots for CETP (TP2 antibody) and calreticulin (Sigma-Aldrich Corp.) were performed as previously described (13).

### Perilipin Western blots

Confluent control and CETP<sup>+</sup> cells were grown in media with or without 200  $\mu\text{M}$  oleate/BSA for 48 h. The cells were washed three times with PBS, harvested in hypotonic lysis buffer, and incubated on ice for 30 min with frequent vortexing. Cells were

passed 10 times through a 26-gauge needle, then spun at 1,000  $g$  for 10 min. Supernatants were collected, their protein content was determined (22), and aliquots containing equal protein were spun at 100,000  $g$  for 1 h. The entire supernatant containing cytosol and lipid droplets was collected. Equal volumes of each sample were cold acetone precipitated and separated on 4–20% SDS-PAGE gel. The levels of perilipin 1, 2, and 3 were determined using 1/2,000 dilutions of rabbit anti-human antibodies against perilipin protein family (PLIN) 1 (Sigma), PLIN2, or PLIN3 (ThermoFisher Scientific, Middletown, VA) followed by horseradish peroxidase-conjugated secondary antibody.

### Diacylglycerol acyltransferase activity

Cells were lysed by sonication and centrifuged at 25,000  $\times g$  as previously described (23). Microsomes were isolated from the resultant supernatant as the 100,000  $\times g$  pellet. Diacylglycerol acyltransferase (DGAT) activity was measured using [ $^{14}\text{C}$ ]oleoyl-CoA (Perkin-Elmer Life Sciences) and *sn*-1,2-dioleoylglycerol (Avanti Polar Lipids) as substrates. Assays were performed as described by Ganji et al. (23) using 20  $\mu\text{g}$  microsomal protein and an incubation time of 30 min. DGAT assays were stopped by lipid extraction (21). TG in the extract was isolated by thin layer chromatography in a developing system of hexanes:diethyl ether:acetic acid (70:30:1), and its  $^{14}\text{C}$  content determined by scintillation counting.

### Real-time quantitative PCR

Total RNA was extracted from control and CETP<sup>+</sup> cells using RNeasy Mini Kit (Qiagen, Germantown, MD) following the manufacturer's protocol. cDNA was generated using a High Capacity RNA-to-cDNA Kit, and real-time quantitative PCR (qPCR) analysis was performed using TaqMan gene expression primer/probe sets and TaqMan Universal Master Mix from Life Technologies. Ribosomal 18S, judged to be the best reference gene among five candidate genes tested, was used to normalize gene expression data. Gene expression was calculated using the  $2^{-\Delta\Delta\text{CT}}$  method (24) and reported relative to control cells.

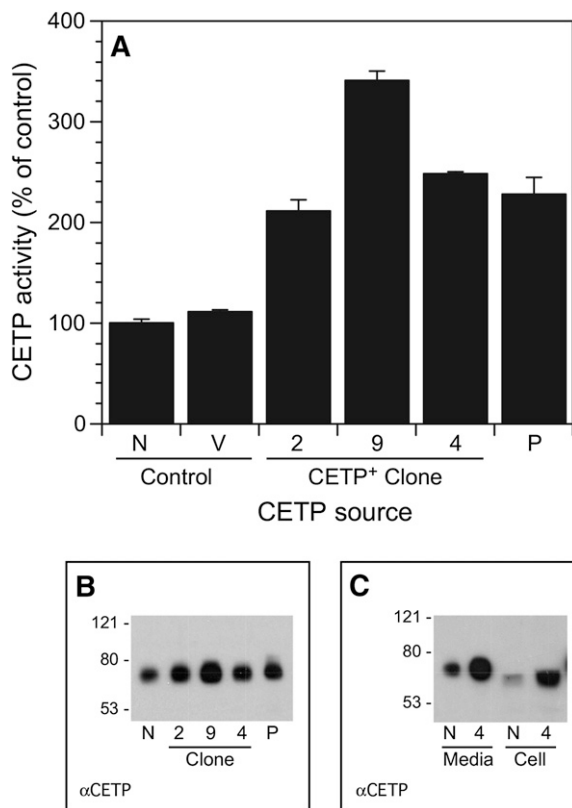
### Analytical methods

Protein was quantified by a modification of the Lowry et al. method (22) with BSA as standard. Cholesterol was determined by an enzymatic kit (ThermoFisher Scientific). TG was quantified by a fluorometric, enzymatic kit from Abcam (Cambridge, MA). Each sample was assayed with and without the addition of lipase; values in the absence of lipase were used as the assay blank for that sample. Lipid phosphorus was quantified by the method of Bartlett (25). Statistical analysis was performed by unpaired *t*-test.

## RESULTS

### Perturbed TG storage in CETP-overexpressing cells

To examine the role of full-length CETP in cellular lipid metabolism, we created SW872 transfectants that stably overexpress this protein. Three clones secreting 2- to 3.4-fold higher CETP activity compared with control cells were isolated (Fig. 1A). Increases in media CETP activity closely mirrored CETP mass (Fig. 1B). The apparent molecular weight of CETP depends on its glycosylation status (6). CETP secreted by all cells was identical in molecular weight to CETP isolated from human plasma (Fig. 1B),



**Fig. 1.** CETP expression by CETP<sup>+</sup> clones. A: CETP activity in media incubated with the indicated cells for 48 h. Lipid transfer activity in control cells was  $19.7 \pm 0.8\%$ /ml ( $t = 16$  h). B: CETP Western blot of the conditioned media assayed in A. In A and B, media aliquots were normalized to the number of cells per well. C: CETP Western blot of conditioned media and cell homogenates (25  $\mu$ g protein). N, native SW872 cells; P, partially purified CETP from plasma; V, vector-transfected SW872 cells. Numerical designations refer to the CETP<sup>+</sup> clone number.

indicating that CETP is properly synthesized even when overexpressed several fold. In addition to increased secretion of CETP, cells overexpressing CETP also have similar increments in intracellular CETP (Fig. 1C). Intracellular CETP had a slightly lower molecular weight, consistent with incomplete carbohydrate side-chain processing as previously reported (6).

Cells deficient in CETP have impaired TG storage capacity (14). To examine the impact of CETP overexpression on TG storage, cells were incubated with 200  $\mu$ M <sup>3</sup>H-oleate/BSA for 48 h. Surprisingly, all clones overexpressing CETP had reduced ability to accumulate TG. This was observed whether CETP<sup>+</sup> clones were compared with a nonclonal population (pool) of vector control cells or individual control clones (Table 1). Similar reductions in TG accumulation were noted in all clones regardless of their extent of CETP overexpression.

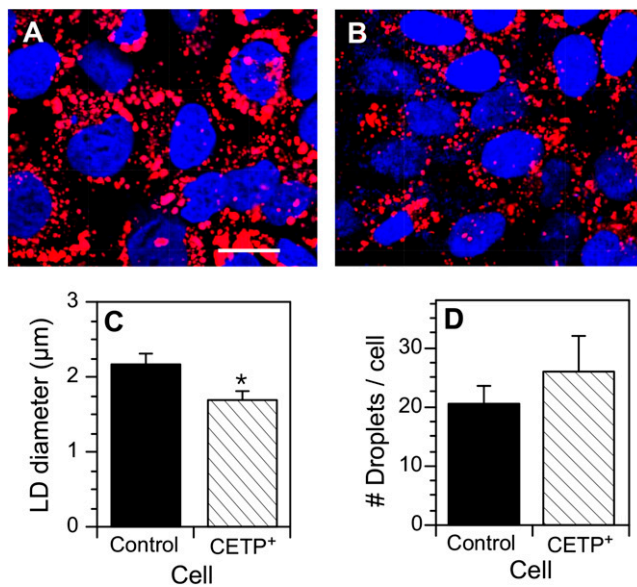
Clone 4 cells were selected for further study to investigate the basis for the decreased TG content. When incubated with oleate/BSA to induce lipid storage droplet formation, these CETP<sup>+</sup> cells had an aberrant lipid droplet phenotype (Fig. 2A, B). Compared with control cells, lipid droplets in CETP<sup>+</sup> cells were significantly smaller in size

TABLE 1. Effect of CETP overexpression on cellular TG content

Cell	TG (nmol/mg Protein)	% of Vector Pool
Control pool	$80.4 \pm 9.5$	100
Control clone 1	$85.8 \pm 17.3$	106.7
Control clone 2	$80.0 \pm 2.0$	99.5
Control clone 3	$86.5 \pm 10.4$	107.6
CETP <sup>+</sup> clone 2	$47.2 \pm 3.0$	58.7
CETP <sup>+</sup> clone 4	$48.9 \pm 6.8$	60.8
CETP <sup>+</sup> clone 9	$55.3 \pm 9.0$	68.8

Vector-transfected SW872 cells (pool and individual clones) and three CETP<sup>+</sup> clones were incubated with 200  $\mu$ M <sup>3</sup>H-oleate/BSA for 48 h to uniformly label the TG pool. TG was extracted, purified by thin layer chromatography, and quantified by scintillation counting. Values are mean  $\pm$  SD,  $n = 3$ . The TG content of all CETP<sup>+</sup> clones differed significantly from control ( $P < 0.05$ ).

(Fig. 2C). CETP<sup>+</sup> cells tended to contain more lipid droplets per cell, but this difference did not reach statistical significance (Fig. 2D). Assuming a spherical lipid droplet shape and considering the number of droplets per cell, the lipid droplet volume in CETP<sup>+</sup> cells was 60% of that in vector control cells ( $66.0 \pm 4.3 \mu\text{m}^3/\text{cell}$  vs.  $109.1 \pm 7.5 \mu\text{m}^3/\text{cell}$ , respectively). This compares very well to that determined by <sup>3</sup>H-oleate incorporation (Table 1). By direct enzymatic assay of TG mass, CETP<sup>+</sup> cells contained  $48 \pm 8\%$  less TG than control. The agreement between <sup>3</sup>H-oleate incorporation and enzymatic assay values on the magnitude of the TG decrease in CETP<sup>+</sup> cells indicates that the 48 h labeling period is sufficient to reach isotopic



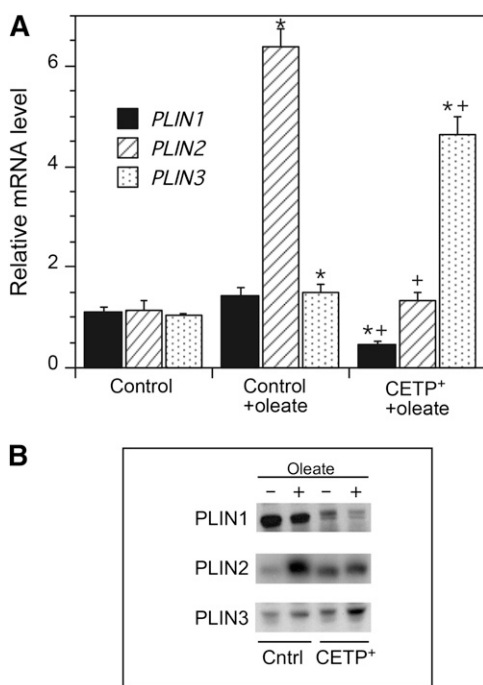
**Fig. 2.** Lipid droplets in vector-transfected SW872 and CETP<sup>+</sup> cells. Cells at 70% confluence were incubated with 100  $\mu$ M oleate/BSA for 24 h to stimulate the development of lipid droplets. Cells were fixed, stained with BODIPY 558/568 C<sub>12</sub> (lipid) and DAPI (nucleus), and viewed by confocal microscopy. The number and size of BODIPY 558/568 C<sub>12</sub> stained lipid droplets were quantified by ImagePro. A: Vector-transfected SW872 cells. Bar = 10  $\mu$ m. B: CETP<sup>+</sup> cells. C: Quantification of lipid droplet (LD) diameter. D: Number of lipid droplets per cell. Data in C and D (mean  $\pm$  SD) were derived from analyzing three fields of both cell types, each field containing 83–105 cells. \*  $P < 0.01$  versus control cells.

equilibrium in the TG pool, just as we have previously reported for CE (13).

To determine whether the smaller lipid droplets in CETP<sup>+</sup> cells were associated with changes in the expression of lipid droplet protein genes, we measured the mRNA levels of *PLIN1*, *2*, and *3*. Oleate treatment of control cells caused a small increase in *PLIN1* and *PLIN3* mRNA, but a 6-fold increase in *PLIN2* expression (Fig. 3A). In contrast, in oleate-treated CETP<sup>+</sup> cells *PLIN1* and *PLIN2* expression was much lower than that in oleate-treated control cells, but *PLIN3* expression was increased more than 4-fold. These mRNA levels also correlate with the cellular levels of PLIN proteins. In the presence of oleate to stimulate lipid droplet formation, only PLIN2 increased significantly in control cells, whereas only PLIN3 was increased by oleate in CETP<sup>+</sup> cells (Fig. 3B). Oleate-treated CETP<sup>+</sup> cells contained markedly less PLIN1 and PLIN2 than oleate-treated control cells, but more PLIN3.

### Differentiation status of CETP<sup>+</sup> cells

During adipogenesis, *CCAAT/enhancer binding protein* (*CEBP*) $\beta$ , *PPAR* $\gamma$ , and *CEBP* $\alpha$  genes are expressed sequentially, leading to upregulation of pathways involved in TG synthesis and storage. The absolute levels of these transcription factors can vary >10-fold during differentiation,

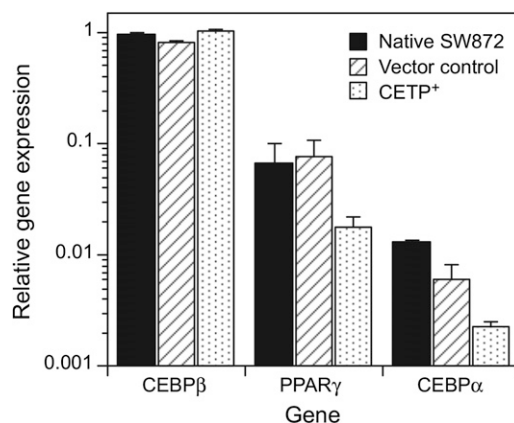


**Fig. 3.** Perilipin gene expression and protein levels. A: Native SW872 and CETP<sup>+</sup> cells were grown in 200  $\mu$ M oleate for 48 h to induce lipid droplet formation. mRNA levels determined by qPCR are shown for *PLIN1*, *PLIN2*, and *PLIN3*. SW872 cells grown in the absence of added oleate were used as reference. Values are the mean  $\pm$  SD of the values determined on RNA isolated from two to three separate cell cultures. \*  $P < 0.05$  versus control; +  $P < 0.05$  versus oleate-treated control. B: Western blot of perilipins in the 100,000  $g$  supernatant isolated from vector-transfected SW872 (Cntrl) and CETP<sup>+</sup> cells. Cells were grown with or without oleate as described in A.

whereas the expression of these genes relative to each other is indicative of differentiation status (26, 27). We initially considered that the reduced TG content of CETP<sup>+</sup> cells might occur because the differentiation status of these cells has been altered. Although we observed differences in the mRNA levels for some of genes, these differences were small compared with the natural variation of these genes during differentiation (Fig. 4). For native SW872, vector control, and CETP<sup>+</sup> cells, the expression of these transcription factor genes followed the same order: *CEBP* $\beta$  > *PPAR* $\gamma$  > *CEBP* $\alpha$ . The expression of *SREBF1*, a master regulator of lipid synthesis, was unchanged, and the expression of *PPAR* $\gamma$  target genes such as *PNPLA2*, *FASN*, and *APOE* (28, 29) was not different in CETP<sup>+</sup> cells (Table 2). *SCL2A4* and *FABP4* levels were reduced by about half. Overall, these data suggest that the differentiation status of CETP<sup>+</sup> cells is very similar to that of control cells.

### TG metabolism in CETP<sup>+</sup> cells

The reduced TG content of CETP<sup>+</sup> cells is partly explained by reduced biosynthesis. TG synthetic rates were determined in cells incubated with <sup>3</sup>H-oleate/BSA over a 4 h time course, during which TG synthesis was linear. In control cells, the TG synthetic rate was  $15.8 \pm 2.8$  nmol/mg protein/h ( $n = 6$ ). In CETP<sup>+</sup> cells, this rate was 26% lower ( $11.6 \pm 2.7$  nmol/mg protein/h ( $n = 6$ ),  $P = 0.03$ ). We also determined whether CETP overexpression affected the movement of newly synthesized TG from the endoplasmic reticulum to lipid droplets during this 4 h period. For both cell types, after an initial 1 h lag, TG accumulation in lipid droplets was nearly linear (data not shown). There was no discernable difference between native control and CETP<sup>+</sup> cells in this interorganelle transfer, showing that CETP overexpression did not appear to stimulate this process. In separate experiments, there was also no difference in the extent to which new TG was moved to lipid droplets in native SW872 versus vector-transfected SW872 cells (data not shown).



**Fig. 4.** Expression of differentiation genes. Native SW872, vector-transfected SW872, and CETP<sup>+</sup> cells were grown to 90% confluence and then harvested to determine the expression levels of genes associated with adipocyte differentiation. mRNA levels were determined by qPCR. Data are presented relative to the expression of *CEBP* $\beta$  in native SW872 cells.

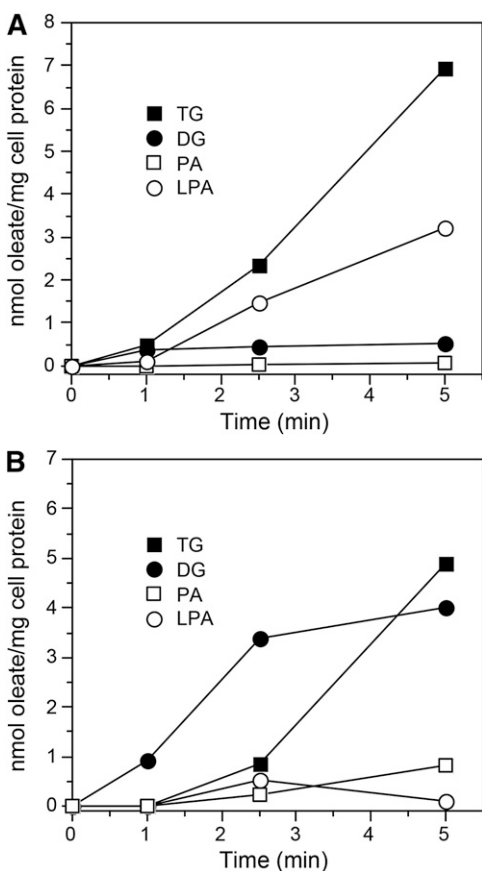
TABLE 2. Expression of adipocyte-related genes

Gene	Control	CETP <sup>+</sup>
<i>SREBF1</i>	1.08 ± 0.05	0.97 ± 0.29
<i>PNPLA2</i>	1.06 ± 0.04	0.91 ± 0.06
<i>SCL2A4</i>	1.16 ± 0.08	0.53 ± 0.34 <sup>a</sup>
<i>FABP4</i>	1.01 ± 0.24	0.40 ± 0.15 <sup>a</sup>
<i>APOE</i>	1.00 ± 0.05	0.89 ± 0.21
<i>FASN</i>	1.13 ± 0.10	2.12 ± 1.10

The expression of the indicated genes was determined by qPCR. Data are the mean ± SD (n = 2–3 separate cell cultures). *FABP4*, fatty acid binding protein 4; *PNPLA2*, adipocyte triglyceride lipase; *SCL2A4*, glucose transporter type 4 (GLUT4); *SREBF1*, sterol regulatory element-binding protein 1.

<sup>a</sup>*P* < 0.05 versus control.

To investigate a mechanistic basis for the lower TG synthesis in CETP<sup>+</sup> cells, we traced the initial movement of <sup>3</sup>H-oleate through the TG biosynthetic pathway. In control cells, TG synthesis was nearly linear over the study time course (Fig. 5A). Diglyceride (DG) levels and phosphatidic acid levels were consistently low, although in some experiments DG levels were high at 1 min and declined quickly thereafter. Lysophosphatidic acid levels tended to increase over the time course, although in some experiments this increase was smaller than shown here. In con-



**Fig. 5.** Incorporation of <sup>3</sup>H-oleate into TG and its precursors. Native SW872 (A) and CETP<sup>+</sup> (B) cells were incubated with 200 μM <sup>3</sup>H-oleate/BSA for the indicated times. Lipids were extracted, fractionated by thin layer chromatography, and <sup>3</sup>H quantified by scintillation counting. PA, phosphatidic acid; LPA, lysophosphatidic acid. Results are representative of four experiments.

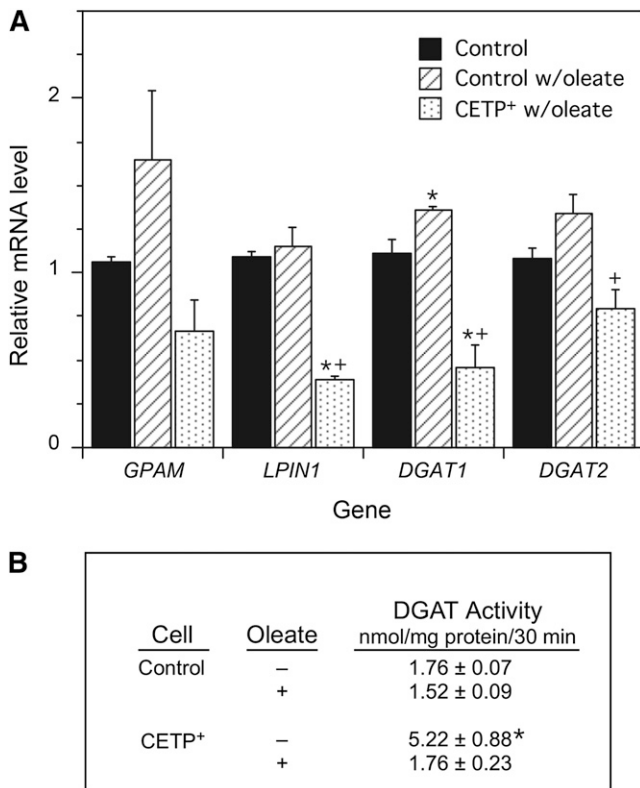
trast, in CETP<sup>+</sup> cells the production of TG lagged initially and remained lower at the latest time point. On the other hand, DG levels were much higher than in control cells at all time points (Fig. 5B). In three experiments, at 5 min the DG/(DG + TG) ratio in CETP<sup>+</sup> cells was 0.46 ± 0.05 compared with 0.17 ± 0.07 in native SW872 cells and 0.23 ± 0.09 in vector-transfected control cells.

In addition to its conversion to TG, DG is also a substrate for neutral phospholipid synthesis. Oleate incorporation into the major membrane phospholipid, phosphatidylcholine, was 2-fold higher in CETP<sup>+</sup> cells (0.59 ± 0.12 vs. 0.25 ± 0.07 nmol/mg protein at 5 min, n = 3, *P* = 0.014). Nonetheless, calculated from the sum of <sup>3</sup>H-oleate incorporated into phosphatidylcholine, TG, and all TG precursors at 5 min, the flux of oleate through this biosynthetic pathway was diminished in cells overexpressing full-length CETP [8.31 ± 1.78 nmol/mg protein vs. 11.96 ± 0.40 nmol/mg protein in control cells (n = 3, *P* = 0.026)].

Expression levels of genes involved in the TG biosynthetic pathway were determined to better understand the cause of this aberrant TG synthesis. Incubation of control cells with oleate induced slight increases in the four genes examined, but only the *DGAT1* increase reached statistical significance (Fig. 6A). In CETP<sup>+</sup> cells, the levels of *GPAM*, whose product mediates the initial and rate-limiting step in TG biosynthesis, were reduced, but this did not quite reach statistical significance (*P* = 0.08). However, mRNA levels of *DGAT2* were reduced by 40%, and *LPIN1* and *DGAT1* were decreased by 65% compared with oleate-treated control cells. *LPIN1*'s gene product, phosphatidate phosphohydrolase, produces DG, and *DGAT1* and *DGAT2* mediate the conversion of DG to TG. The impaired conversion of DG into TG and the reduced levels of *DGAT1* and *DGAT2* mRNA suggest that DGAT activity might be reduced in CETP<sup>+</sup> cells. However, this was not the case. In the absence of oleate, DGAT activity in the microsomal fraction from CETP<sup>+</sup> cells was increased >2-fold, but under conditions where TG deposition is stimulated (+oleate), DGAT activity in CETP<sup>+</sup> cells was not different from control (Fig. 6B).

### Fatty acid uptake

Another potential mechanism for the reduced TG content of CETP<sup>+</sup> cells is impaired fatty acid uptake. When quantified, we found that oleate uptake by CETP<sup>+</sup> cells was, on average, 24% lower than in control cells (Fig. 7). One mechanism for fatty acid uptake is through the *CD36* scavenger receptor (30). Knockout of the *CD36* gene markedly impairs the accumulation of TG in adipose (31). We observed that mRNA levels for *CD36* were reduced by 60% in CETP<sup>+</sup> cells (0.40 ± 0.20 vs. 1.12 ± 0.07, n = 3, *P* < 0.05). The incubation of control cells in oleate-containing media for 48 h to promote TG accumulation increased *CD36* expression 2.8-fold (2.78 ± 0.51, *P* < 0.05). In contrast, *CD36* expression in CETP<sup>+</sup> cells was not responsive to oleate treatment (0.29 ± 0.06), causing *CD36* expression in CETP<sup>+</sup> to be about 10% of control values under conditions where TG synthesis is stimulated. Reduced fatty acid

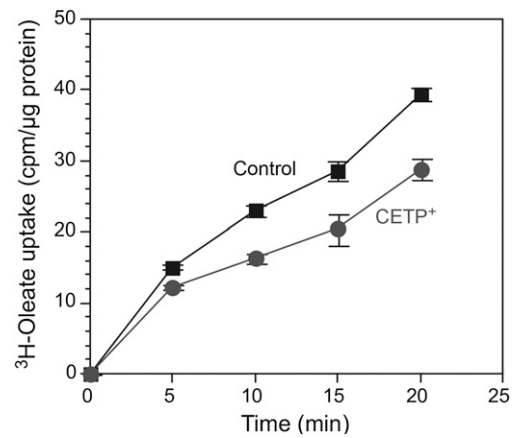


**Fig. 6.** mRNA levels of TG biosynthetic pathway enzymes and DGAT activity. **A:** Control and CETP<sup>+</sup> cells were incubated without or with 200  $\mu$ M oleate for 48 h, then mRNA levels of *GPAM* (glycerol-3-phosphate acyltransferase 1, mitochondrial), *LPIN1* (phosphatidate phosphatase), and *DGAT1* and 2 were measured by qPCR. Data are expressed relative to the gene levels in SW872 cells incubated without oleate. Values are the mean  $\pm$  SE of the values determined on RNA isolated from two to three separate cell cultures. \*  $P < 0.05$  versus control; +  $P < 0.05$  versus oleate-treated control. **B:** DGAT activity in isolated microsomes. Data are the mean  $\pm$  SD of values determined on two separate microsomal preparations for each cell type, each assayed in triplicate. \*  $P < 0.05$  versus all other values.

uptake could contribute to the decreased TG content of CETP<sup>+</sup> cells.

#### Cellular localization of CETP

We previously suggested that one of the functions of intracellular CETP might be the interorganelle transfer of lipids, giving it a possible direct role in lipid droplet formation (14). Such a function requires that at least a portion of cellular CETP be in the cytoplasm where it can perform this transfer function, instead of being in the endoplasmic reticulum lumen, the expected location for a protein in the secretory pathway. We took advantage of the higher intracellular CETP levels in CETP<sup>+</sup> cells to investigate the location of CETP in cells. Confocal imaging showed that CETP is widely distributed within cells (**Fig. 8A**) with a cellular distribution similar to that of the endoplasmic reticulum protein calnexin (**Fig. 8B**). While there is considerable colocalization of CETP with the endoplasmic reticulum, in peripheral areas where lipid droplets are abundant CETP immunofluorescence greatly exceeded that of the endo-



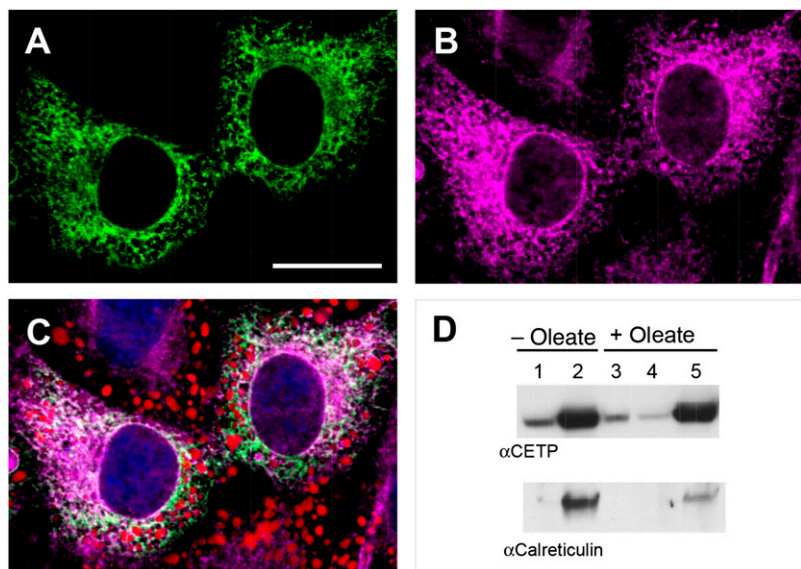
**Fig. 7.** Oleate uptake. Native SW872 and CETP<sup>+</sup> cells were incubated with 200  $\mu$ M <sup>3</sup>H-oleate/BSA for the indicated time, and then the cellular content of <sup>3</sup>H was determined. Values are the mean  $\pm$  SD of triplicate determinations.

plasmic reticulum marker (**Fig. 8C**). This suggests that some CETP may not be associated with endoplasmic reticulum in this region.

In plasma, almost all CETP is bound to lipoproteins (32, 33) even though during lipid transfer it carries lipids between lipoproteins (34, 35). This suggests that any CETP released from the endoplasmic reticulum into the cytoplasm might be membrane bound. Because lipoprotein-bound CETP is dissociated by centrifugation (36), we reasoned that CETP not contained within the endoplasmic reticulum lumen can be identified by cell fractionation studies. Lysed cells were centrifuged to yield an endoplasmic reticulum-containing membrane fraction, cytosol, and lipid droplets. Calreticulin, a soluble endoplasmic reticulum luminal protein, was used to verify the integrity of the endoplasmic reticulum. Regardless of oleate treatment, most CETP was associated with the endoplasmic reticulum membrane fraction (**Fig. 8D**). However, a subfraction of CETP was found in the cytosolic fraction under conditions where calreticulin was detected only in the membrane-rich fraction. Moreover, when cells were pretreated with oleate to stimulate lipid droplet formation, a small CETP pool was also found in the lipid droplet fraction. Collectively, these immunolocalization and cell fractionation studies show that most cellular CETP is associated with the endoplasmic reticulum, but a portion of intracellular CETP is in the cytoplasm and may associate with lipid droplets.

#### Turnover of TG in CETP-overexpressing cells

In CETP<sup>+</sup> cells, the decrease in TG synthesis accounted for about half of the decline in TG content, suggesting that other mechanisms contribute to the reduced capacity of these cells to store TG. We assessed the role of TG hydrolysis in the reduced TG content of CETP<sup>+</sup> cells by pre-incubating cells with <sup>3</sup>H-oleate to label TG pools, then placing them in oleate-free chase media containing albumin and a fatty acyl-CoA synthetase inhibitor to prevent reincorporation of <sup>3</sup>H-oleate once cleaved from TG.

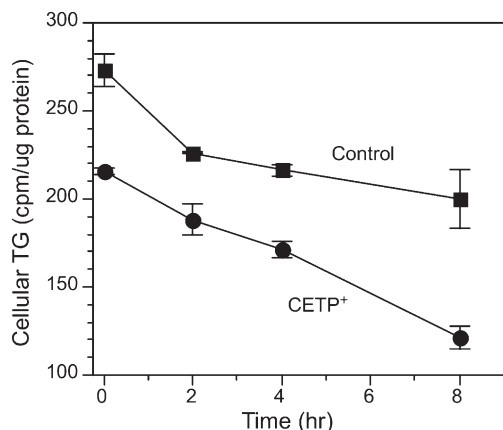


**Fig. 8.** Immunolocalization of CETP. CETP<sup>+</sup> cells were grown in the presence of 100  $\mu$ M oleate/BSA for 48 h to induce lipid droplet formation. A, B: Cells were fixed and immunostained for CETP (A) and calnexin (B). Bar = 20  $\mu$ m. C: Merge of CETP (green), calnexin (magenta), and BODIPY lipid stain (red). Confocal microscopy images are shown. D: CETP<sup>+</sup> cells grown without or with 500  $\mu$ M oleate were lysed and cellular components fractionated by centrifugation into cytosol (lanes 1 and 4), endoplasmic reticulum-containing membrane (lanes 2 and 5), and lipid droplet (lane 3) fractions. CETP and calreticulin were detected by Western blot.

Although control cells had a more rapid initial loss of <sup>3</sup>H-TG in the first 2 h, the loss of <sup>3</sup>H from the TG pool was nearly linear for both cell types thereafter (**Fig. 9**). Over the 2–8 h period, <sup>3</sup>H-TG hydrolysis in CETP<sup>+</sup> cells was 2.7-fold higher than control cells (11.4 vs. 4.3 cpm/ $\mu$ g protein/h, respectively). Even including the initial 2 h period, CETP<sup>+</sup> cells lost 44% of their TG pool over 8 h, whereas control cells hydrolyzed only 27%. These results show that the absolute and fractional hydrolysis rates of lipid droplet TG are elevated in cells overexpressing CETP.

#### Effect of CETP overexpression on cholesterol metabolism

The reduced TG content of cells overexpressing CETP mirrors the decreased TG content of cells with low CETP expression (14). Because CETP-deficient cells also have significant alterations in cholesterol metabolism, we investigated the effect of CETP overexpression on CE levels and CE synthetic rates in oleate-treated



**Fig. 9.** TG hydrolysis. Native SW872 and CETP<sup>+</sup> cells were incubated in 200  $\mu$ M <sup>3</sup>H-oleate/BSA for 24 h. After washing in oleate-free media, cells were incubated in media containing 100  $\mu$ g/ml HDL, 0.01% BSA, and 10  $\mu$ M Triacsin C. Values are the mean  $\pm$  SD of triplicate determinations.

cells. CETP<sup>+</sup> cells had 31% lower CE content compared with control cells even though the CE synthetic rate was 50% higher (**Table 3**). This paradoxical observation is explained by the markedly elevated hydrolysis of the CE pool in CETP<sup>+</sup> cells (**Table 3**). Thus, CETP<sup>+</sup> cells have elevated CE synthesis but also increased degradation leading to reduced CE content. This differs from CETP-deficient cells, which have elevated CE content (13, 14), decreased CE synthesis [ $0.12 \pm 0.05$  nmol/mg protein/h ( $n = 4$ ),  $P = 0.04$  vs. control (**Table 3**)], and impaired CE hydrolysis (14).

In addition to altering CE levels and the dynamics of this lipid pool, CETP overexpression also altered genes involved in sterol homeostasis. Compared with control cells, *HMGCR* mRNA levels were increased more than 2-fold in CETP<sup>+</sup> cells (**Table 3**) but unchanged in CETP-deficient cells ( $0.90 \pm 0.18$  relative units). However, mRNA levels for the intracellular cholesterol transporter *ABCG1* (37) were profoundly decreased in both CETP<sup>+</sup> (**Table 3**) and CETP-deficient cells ( $0.14 \pm 0.04$  relative units). Together, these findings show that CETP overexpression induces a unique cholesterol phenotype.

## DISCUSSION

We previously reported that chronic inhibition of both CETP isoforms in SW872 cells disrupted cholesterol homeostasis, increased CE content, and decreased TG content. These changes were associated with decreased hydrolysis and release of these lipids from lipid droplets under conditions stimulating efflux and the inefficient transport of CE and TG from the endoplasmic reticulum to lipid droplets (14). In vitro, we demonstrated that CETP can promote this interorganelle lipid movement, suggesting that the inefficient transport of CE and TG in CETP-deficient cells was directly related to reduced CETP levels.

To further understand the involvement of CETP in cellular lipid metabolism, we investigated the effects of



TABLE 3. Sterol metabolism in native and CETP<sup>+</sup> cells

Cell	CE content	CE synthesis	CE hydrolysis	Relative mRNA level	
				<i>HMGCR</i>	<i>ABCG1</i>
Control	2.81 ± 0.41	0.22 ± 0.07	23.0 ± 1.0	1.07 ± 0.08	1.06 ± 0.07
CETP <sup>+</sup>	1.93 ± 0.31 <sup>a</sup>	0.33 ± 0.03 <sup>b</sup>	59.1 ± 10.3 <sup>b</sup>	2.59 ± 0.35 <sup>b</sup>	0.01 ± 0.01 <sup>b</sup>

CE content was determined from the steady-state level of <sup>3</sup>H-CE after 48 h incubation with 200 μM <sup>3</sup>H-oleate/BSA. CE synthesis rates were determined from the slope of the <sup>3</sup>H-CE accumulation curve over 4 h. To measure CE hydrolysis, after cells were incubated with <sup>3</sup>H-oleate/BSA for 48 h, the decay in the <sup>3</sup>H-CE pool was measured after oleate removal and the addition of HDL and BSA to the media. Gene expression for *HMGCR* (HMG-CoA reductase) and *ABCG1* was measured by qPCR. Units are CE content, nmol/mg protein (n = 3); CE synthetic rate, nmol/mg protein/h (n = 6); CE hydrolysis, % decrease in CE pool over 8 h period (n = 2-3).

<sup>a</sup>P < 0.05 versus control.

<sup>b</sup>P < 0.01 versus control.

overexpressing one of the two CETP isoforms present in cells. Stable cell clones synthesizing 2- to 3.4-fold more full-length CETP than control were produced. These elevated levels are within the range of enhanced CETP secretion that occurs when SW872 and Caco2 cells are treated with acetylated LDL (13) or when animals consume cholesterol-enriched diets (38). All three clones isolated were defective in their ability to store TG, accumulating ~40% less TG regardless of CETP overexpression level. Lipid droplets that accumulated in cells overexpressing CETP were much smaller but as numerous as those in control cells. Reduced TG storage and smaller lipid droplets are also seen in vivo in the adipocytes from CETP transgenic mice expressing the transgene under control of an adipose tissue-specific promoter (15). Collectively, these data suggest that decreased TG content is a common phenotype induced when CETP expression is increased.

Our data suggest two likely causes for the reduced TG content of cells overexpressing CETP. First, the flux of fatty acid through the TG biosynthetic pathway is reduced. Although phospholipid synthesis was increased in CETP<sup>+</sup> cells, which diverts DG for phospholipid instead of TG synthesis, this pathway is quantitatively small and, therefore, does not explain the reduction in TG synthesis. Further, CETP<sup>+</sup> cells have impaired conversion of DG to TG. mRNA levels for the enzymes responsible for this conversion, *DGAT1* and *DGAT2*, are decreased up to 60%; however, direct assay of total cellular DGAT activity did not reveal any decrease. CD36 is a plasma membrane receptor that binds multiple ligands including fatty acids (30, 39). Recent studies have suggested that CD36 does not directly transport fatty acids across the plasma membrane, but rather through signaling events or direct interaction with enzymes such as acyl-CoA synthetase it enhances fatty acid uptake and promotes TG synthesis (40). *CD36* mRNA levels were reduced by 90% in CETP<sup>+</sup> cells grown in oleate-containing media, suggesting that reduced fatty acid uptake may explain the reduced flux of fatty acids through the TG biosynthetic pathway. Indeed, we determined that oleate uptake by CETP<sup>+</sup> cells was reduced by 24%. This value is nearly identical to the reduction in fatty acid flux through the TG synthetic pathway determined in short-term assays and to the TG synthetic rate measured in longer-term assays. Additionally, because reduced *CD36*

expression has been shown to impair the conversion of DG to TG (31), the very low expression of CD36 in CETP<sup>+</sup> cells may explain the unusual DG kinetics observed in these cells. Overall, these data suggest that reduced fatty acid uptake, perhaps secondary to aberrant *CD36* expression, contribute to the deficits noted in TG synthesis.

The second mechanism identified as causative in the low TG content of CETP<sup>+</sup> cells is elevated catabolism of this lipid. The turnover of TG stored in lipid droplets was more than 2.5-fold higher in cells overexpressing CETP. It seems likely that this elevated breakdown of stored TG is related to changes in the nature of lipid droplets that accumulate in CETP<sup>+</sup> cells. CETP<sup>+</sup> cells synthesize normal numbers of lipid droplets; however, these droplets are significantly smaller in size. Quantification of *PLIN* mRNAs and direct assessment of perilipin protein levels in the 100,000 g supernatant of cells show that the small lipid droplet phenotype correlates with aberrantly high levels of PLIN3 and low PLIN 1 and PLIN2 levels. This PLIN protein profile is characteristic of immature lipid droplets (41). Higher turnover rates for small lipid droplets are commonly observed in other systems (42, 43). In this context, the elevated phosphatidylcholine synthesis observed in CETP<sup>+</sup> cells may also contribute to the formation of small-sized lipid droplets in these cells. Phosphatidylcholine synthesis stabilizes the lipid droplet surface and decreases lipid droplet growth by fusion, resulting in smaller lipid droplets (44). Collectively, these results suggest that full-length CETP overexpression does not interfere with mechanisms involved in lipid droplet initiation, but it does interfere with their maturation to larger lipid-filled structures. Whether enhanced TG catabolism causes lipid droplets to be small, or whether TG catabolism is elevated because small lipid droplets are inherently more metabolically active remains to be determined.

Although both full-length CETP overexpression and the simultaneous suppression of full-length and exon 9-deleted CETP synthesis (14) cause cells to accumulate less TG, the effect of these manipulations on CE metabolism were different. Cell with reduced CETP expression contain high CE levels, which resulted from elevated CE synthesis and reduced CE hydrolysis (14). Conversely, we observed that cells overexpressing full-length CETP contain less CE. This reduction is largely due to a 2.5-fold

increase in the catabolism of CE. Thus, the effects of CETP over- or underexpression on CE levels are opposite and largely explained by the different effects these two conditions have on CE hydrolysis rates.

Subsequent to synthesis, TG and CE must move from the endoplasmic reticulum to the lipid droplet for storage. Several mechanisms for this process have been proposed (43). According to a prevailing model, nascent lipid droplets form by budding of the cytoplasmic face of the endoplasmic reticulum membrane and then mature in the cytoplasm either by fusion of small droplets or by the transfer of lipids into existing nascent droplets (41, 45–48). The relative contribution of these two processes to lipid droplet growth is unknown, but there are instances where lipid transfer predominates (41, 42, 47). Multiple proteins appear to be involved in the recruitment of TG into lipid droplets in adipocytes, such as fat storage-inducing transmembrane 2 and fat specific protein 27 (49, 50). We propose in CETP-expressing species that CETP also participates in this process, and our previous studies in CETP-deficient cells support this hypothesis (14). Our findings here show that CETP overexpression did not enhance the rate of TG transfer to lipid droplets, suggesting that endogenous CETP levels are sufficient for this process to occur. We also found that intracellular CETP is distributed throughout the cell. Much of this CETP appears to associate with the endoplasmic reticulum, consistent with a previous observation that intracellular CETP is susceptible to endoglycosidase H digestion (6). However, a CETP pool not associated with the endoplasmic reticulum is present in the cellular region where lipid droplets accumulate. This may be the pool of CETP we determined to be cytosolic in cell fractionation studies. Although the mechanism for CETP release into the cytoplasm is unknown, the escape of other endoplasmic reticulum luminal proteins to the cytoplasm has been reported (51–53). Notably, just like CETP, several of these proteins bind to plasma lipoproteins. Perhaps their amphipathic nature is involved in the release mechanism.

CETP expression is upregulated early in adipocyte development (54, 55). Additionally, the relative abundance of full-length and exon 9-deleted CETP mRNAs is variable and tissue specific (6). These data suggest that the level of CETP and the ratio of its isoforms reflect specific cellular needs. The data reported here show that overexpression of full-length CETP in SW872 adipocyte-like cells produces a unique lipid metabolic phenotype where TG and CE levels are reduced and cells accumulate small lipid droplets. The reduction in TG results from at least two factors, reduced synthesis, most likely from impaired fatty acid uptake, and enhanced turnover of lipid droplet TG. We hypothesize that overexpressing full-length CETP interferes with normal processes that couple TG homeostasis with the maturation of nascent lipid droplets. As a result, these cells continue to produce nascent lipid droplets normally, but these droplets are underfilled due to underlying disruptions in TG synthesis and degradation. It is notable that both the overexpression of full-length CETP reported here and the suppression of both full-length and

exon 9-deleted CETP isoforms (14) result in the same low TG phenotype. In contrast to CETP<sup>+</sup> cells, the low TG content of CETP-deficient cells is caused by reduced TG synthesis and inefficient transport of newly synthesized TG out of the endoplasmic reticulum, which appears to lead to its premature degradation before incorporation into lipid droplets (D. J. Greene, L. Izem, and R. E. Morton, unpublished observations). It remains to be determined whether the low TG in these two cell models arises from variations in the absolute levels of full-length CETP per se or to a disruption in the cellular levels and the relative amounts of the two CETP isoforms. Overall, these data provide further evidence that endogenous CETP plays a fundamental role in cellular lipid homeostasis in cells naturally expressing this protein. **FF**

#### Note added in proof

During proof stage, the authors informed *the Journal* that an author, Katarzyna Bialkowska, was inadvertently omitted from the first published version of their manuscript.

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