POSSIBLE ROLE FOR INTRACELLULAR CHOLESTERYL ESTER TRANSFER PROTEIN IN ADIPOCYTE LIPID METABOLISM AND STORAGE*

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Running Title: CETP deficiency impairs lipid storage in SW872 adipocytes

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Cholesteryl ester transfer protein (CETP) transfers cholesteryl ester (CE) and triglyceride (TG) between lipoproteins in plasma. However, short-term suppression of CETP biosynthesis in cells alters cellular cholesterol homeostasis, demonstrating an intracellular role for CETP as well. The consequences of chronic CETP deficiency in lipid-storing cells normally expressing CETP have not been reported. Here, SW872 adipocytes stably expressing antisense CETP cDNA and synthesizing 20% of normal CETP were created. CETP-deficient cells had 4-fold more CE but a ~3-fold decrease in cholesterol biosynthesis. This phenotype of cholesterol overload is consistent with the observed 45% reduction in LDL receptor and 2.5-fold increase in ABCA1 levels. However, cholesterol mass in CETP-deficient adipocytes was actually reduced. Strikingly, CETP-deficient adipocytes stored <50% of normal TG, principally reflecting reduced synthesis. The hydrolysis of cellular CE and TG in CETP-deficient cells was reduced by >50%, although hydrolase/lipase activity was increased 3-fold. Notably, the incorporation of recently synthesized CE and TG into lipid storage droplets in CETP-deficient cells was just 40% of control, suggesting these lipids are inefficiently transported to droplets where the hydrolase/lipase resides. The capacity of cellular CETP to transport CE and TG into storage droplets was directly demonstrated in vitro. Overall, chronic CETP deficiency disrupts lipid homeostasis and compromises the TG storage function of adipocytes. Inefficient CETP-mediated translocation of CE and TG from the endoplasmic reticulum to their site of storage may partially explain these defects. These studies in adipocytic cells strongly support a novel role for CETP in intracellular lipid transport and storage.

Cholesteryl ester transfer protein (CETP)\(^1\) \((1)\) mediates the transfer of cholesteryl ester (CE) and triglyceride (TG) among plasma lipoproteins \((2-4)\). CETP is a member of the lipid transfer / lipopolysaccharide binding protein gene family that is composed of highly hydrophobic proteins involved in lipid binding and transport \((5,6)\). Two CETP transcripts exist, a 1.6 kb transcript produced from the full-length, 16 exon CETP gene and a 1.4 kb transcript lacking exon 9 \((180\) nucleotides) derived by alternative splicing \((7)\). mRNAs for both variants are present in all human tissues expressing CETP. Compared to the full-length form, which accounts for essentially all plasma CETP, exon 9-deleted CETP is poorly secreted and its function is unknown \((7,8)\). Exon 9-deleted CETP retains the amino acids that constitute the binding sites for TG and CE \((residues 470-475)\) \((9,10)\) and the bulk of the putative lipoprotein-binding region \((11,12)\). Absence of a portion of this binding region in exon 9-deleted CETP may explain its poor interaction with HDL \((8)\), and lead to a variant with different substrate preference or affinity.

In addition to its abundant hepatic expression, CETP is synthesized by many tissues, such as adipose, adrenal \((7,13-15)\) and tissue macrophages \((16,17)\), that are active in lipid synthesis and/or storage. Both forms of CETP are readily detected in tissue homogenates \((7,8)\), indicating that even the well-secreted full-length form of CETP can accumulate intracellularly. CETP has broad specificity for membrane surfaces and can transfer lipids from native lipoproteins, from liposomes and between membranes \((18,19)\). It appears that
the only essential substrate requirement for CETP activity is a phospholipid surface (20).

There is growing evidence that CETP has intracellular functions as well. For example, the selective uptake of CE from HDL into adipocytes cells is mediated by CETP produced and associated with these cells (21), and adenovirus-mediated CETP expression in mouse hepatocytes directly mediates CE selective uptake from HDL (22). Transient expression of recombinant CETP in COS-7 cells stimulates their ability to efflux cholesterol to HDL by a process that is not inhibited by extracellular anti-CETP antibody or stimulated by the addition of CETP to the culture media (17). And, acute suppression of CETP biosynthesis in HepG2 cells alters multiple aspects of reverse cholesterol transport (23,24). Additionally, our laboratory has shown that CETP gene expression and cholesterol homeostasis are tightly linked (25). Using an antisense oligonucleotide approach, we observed that short-term, partial CETP suppression in the SW872 adipocytic cell line led to a 2-3 fold increase in cellular CE and interfered with the capacity of these cells to efflux cholesterol to an acceptor.

The consequences of long-term CETP deficiency in lipid-storing cells that normally express CETP, such as the human adipocyte, has not been reported. We show here that chronic CETP deficiency induces dramatic changes in the ability of SW872 adipocytic cells to synthesize and store cholesterol and TG. We also demonstrate that these abnormalities in cellular lipid homeostasis can be attributed, at least in part, to an inappropriate cellular distribution of CE and TG, suggesting that CETP may be directly involved in transferring these lipids from the site of their synthesis to their site of storage in SW872 adipocytes.

EXPERIMENTAL PROCEDURES

Materials - The human lipocarcinoma cell line, SW872 (HTB-92), was purchased from American Type Culture Collection (Manassas, VA). Penicillin, streptomycin, bovine serum albumin and sodium oleate were from Sigma (St. Louis, MO). [1α,2α(n)-3H]cholesterol, [9,10(n)-3H]oleic acid, [1-14C]oleic acid and [1-14C]acetic acid sodium salt were from Perkin-Elmer Life Sciences (Boston, MA). EDTA-free protease inhibitor cocktail (cat. #1873580) was from Roche Applied Science (Indianapolis, IN). Immobilized Protein A was from Pierce Chemical (Rockford, IL). LDL, and HDL were prepared from human plasma by sequential ultracentrifugation (26). Lipoprotein deficient-serum (LPDS) was isolated from fresh human serum as the d>1.25 g/ml density fraction. LDL, HDL and LPDS were dialyzed extensively versus 0.9% NaCl, 0.02% EDTA, then sterile filtered.

Preparation of stable transfectants - SW872 adipocytes were cultured in Dulbecco modified Eagle’s medium/Ham’s F-12 (3:1) (DMEM/F-12) containing 10% fetal bovine serum (FBS) (Bio Whitaker, Walkersville, MD) and 50 μg/ml penicillin/streptomycin in 5% CO2/95% air at 37°C. A 549 bp fragment of human CETP cDNA, corresponding to amino acid 1 – 183 of the mature protein (13), was excised from pCETP.11 (American Type Culture Collection #59792) using natural restriction sites in the vector (EcoR I) and the CETP cDNA (Bgl II). This sequence exists in both full-length and exon 9-deleted CETP mRNA. The EcoR I – Bgl II restriction fragment was inserted in reverse orientation into pcDNA3 (Invitrogen, Carlsbad, CA) using BamH I and EcoR I restriction sites in the vector. Sequence was verified by automated sequencing. Vector containing antisense-CETP or vector alone was transfected into SW872 adipocytes using lipofectamine according to manufacturer’s protocol. After a 5 hr transfection, the plasmid-containing media was removed and cells maintained in serum-containing medium overnight. Subsequently, cells were passaged and selection with 500 μg/ml Geneticin (Invitrogen) begun. After 10-15 days, clones were picked and subcultured under the same selection conditions. Confluent cultures of cells were assayed for CETP expression by determining CETP activity and mass (Western blot) in 48-hr conditioned medium collected in the absence of serum. A vector-transfected clone with the same level of CETP as cells transfected with lipofectamine alone was designated as control.

Secreted CETP activity and mass - When cells were nearly confluent, the culture media was removed and fresh serum-free media was added. After 48 hr, the conditioned media was collected, centrifuged at 3000 rpm for 15 minutes to remove
cellular debris and then assayed for CE transfer activity as described (27), with [3H]CE-LDL (donor) and HDL (acceptor) at 10 µg/ml of cholesterol each. In some instances, samples were pre-incubated with 10 µg of anti-CETP IgG (TP2) for 30 min before initiating the transfer assay. CETP secreted into the medium was immunoprecipitated with polyclonal antibody against human CETP (28) and quantified by western blot analysis as previously described (25).

**Incorporation of acetate into cholesterol and CE** - To assess the impact of CETP reduction on the net balance of synthetic and degradative pathways for cholesterol and CE, control cells and CETP-deficient cells were pre-treated with DMEM/F-12 ± 5% LPDS overnight then incubated with DMEM/F-12 supplemented with 5% LPDS or 10% FBS and 0.5 µCi [14C]acetate for 4 days. The cells were washed three times with PBS, and lipids were extracted (29) and fractionated by thin layer chromatography using a mixture of hexanes/diethyl ether/acetic acid (70:30:1). Radiolabeled CE, and free cholesterol were scraped from the plate and counted. Results were not qualitatively different when the cell incubation media contained 200 µM acetate in addition to the tracer. The rate of cholesterol de novo synthesis was similarly determined but under conditions (t ≤ 3 hr) where acetate incorporation was linear.

**CE synthesis and hydrolysis** - To assess the effect of CETP expression on CE metabolism, cells were grown to 80% confluence in media containing 10% FBS then switched to serum-free media containing 1% BSA and 100 µM [3H]oleate/BSA (7:1, mole/mole; 1.2 x 10⁶ cpm/nmole oleate) for 48 hr to prelabel the CE pool. Subsequently, the media was removed, cells washed, and then incubated in serum-free media supplemented with 1% BSA, 100 µM [14C]oleate/BSA (3.5 x 10⁵ cpm/nmole oleate) and 100 µg/ml HDL. At the times indicated, media was removed, cells washed, and the ³H and ¹⁴C content of the cellular CE pool was measured as described above to quantify CE hydrolysis and synthesis reactions, respectively. The rate of CE synthesis from cholesterol, i.e., acylCoA:cholesterol acyltransferase (ACAT) activity, was determined in a similar fashion. Here, after near confluence, cells were incubated in media containing 10% FBS and 500 µM [³H]oleate/BSA. After 5 hr at 37°C, cells were harvested, lipids extracted, and [³H]CE quantified as above.

**Total cholesterol mass in cellular fractions** - Cells were grown in 10% FBS containing medium until they were confluent then incubated either with serum-containing medium or with medium containing 5% LPDS for 48 hr. The cells were washed, resuspended in PBS, disrupted in a glass/teflon homogenizer, and an aliquot was used to measure total cholesterol using a fluorometric assay (30).

**Mass of TG and its synthesis and turnover** - Cells grown to confluence in serum-containing media were incubated for 48 hr in the same media or media supplemented with 500 µM oleate/BSA. The cells were harvested, suspended in PBS and an aliquot was used to measure TG mass using a fluorometric assay (31). To measure the rate of TG synthesis, cells were incubated for the indicated times with DMEM/F-12 media containing 10% FBS plus 100 µM [³H]oleate/BSA. Cells were washed with PBS, scraped, lipids extracted (29), and the content of radiolabeled TG determined as described above for CE. TG hydrolysis activity was measured as described above for CE except that TG-labeled cells also received Triacsin D (12 µM) to block oleate reesterification.

**Oil Red O staining** - Cells were cultured on glass cover slips in 12 wells plate in DMEM/F12 containing 10% FBS and 100 µg/ml penicillin/streptomycin. At 70% confluence, 250µM oleic acid/BSA was added to the cells in culture media containing 2% serum and incubated for 48 hours. Cells were washed 3 times with PBS, fixed in 4% paraformaldehyde solution for 30 min, washed with PBS for 1 min, and incubated in 60% isopropanol for 2 min. The cells were then incubated for 15 min in 0.3% Oil Red O in 60% isopropanol. After distaining for 1 min in 60% isopropanol and washing with water for 10 min, cover slips were mounted on glass slides using DAPI-containing mounting solution. Confocal images were obtained using a Leika TCS-NT confocal laser-scanning microscope. Quantitative analysis of the size of lipid droplets was performed using ImagePro software (Media Cybernetics, Silver Spring, MD).

**Cellular fractionation** - Cell fractionation was done as previously described (32). Briefly con-
fluent cells were harvested and lysed in hypotonic medium (100 mM Tris, pH 7.4, 1 mM EDTA, 10 mM sodium fluoride and protease inhibitor cocktail) for 10 min at 4°C, followed by 10 strokes in a teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 1000 g at 4°C and the supernatant was adjusted to 35% sucrose and layered over 0.5 ml of 50% sucrose. A linear gradient of 0-30% sucrose was layered over the density-adjusted supernatant and centrifuged for 4 hr at 154,000 g at 4°C. Ten fractions of 1 ml each were collected starting from the top of the tube. Immunoblots using an antibody against perilipin, a lipid storage droplet marker, (a generous gift from Dr. Dawn Brasaemle, Department of Nutritional Sciences, Rutgers), or against calnexin, an ER integral membrane protein (Santa Cruz Biotech., Inc., Santa Cruz, CA) were performed to determine the localization of these organelles in the gradient.

To determine the distribution of recently synthesized lipids among cellular fractions, cells in 100 mm dishes were grown in 5 ml media containing 10% FBS and 2 Μ 14C-acetate for 3 days before being homogenized and fractionated by the sucrose gradient procedure above. Lipids in each fraction were extracted (29), separate by thin layer chromatography as above, and quantified by liquid scintillation counting.

Interorganelle lipid transfer assay - Confluent CETP-deficient cells were incubated with DMEM/F12 2% FBS containing 100 μM 3H-oleic acid-BSA for 48 hours before being homogenized and fractionated by the sucrose gradient procedure above. The homogenate was centrifuged for 10 min at 1000 g at 4°C, and the supernatant was adjusted to 35% sucrose and layered over 0.5 ml of 50% sucrose. A linear gradient of 0-30% sucrose was layered over the density-adjusted supernatant and centrifuged for 4 hr at 154,000 g at 4°C. Ten fractions of 1 ml each were collected starting from the top of the tube. Immunoblots using an antibody against perilipin, a lipid storage droplet marker, (a generous gift from Dr. Dawn Brasaemle, Department of Nutritional Sciences, Rutgers), or against calnexin, an ER integral membrane protein (Santa Cruz Biotech., Inc., Santa Cruz, CA) were performed to determine the localization of these organelles in the gradient.

To determine the distribution of recently synthesized lipids among cellular fractions, cells in 100 mm dishes were grown in 5 ml media containing 10% FBS and 2 μCi [14C]acacetate for 3 days before being homogenized and fractionated by the sucrose gradient procedure above. Lipids in each fraction were extracted (29), separate by thin layer chromatography as above, and quantified by liquid scintillation counting.

Neutral cholesteryl ester hydrolase assay - To quantify cellular neutral CE hydrolase activity, confluent cells, incubated with media with or without 10% FBS, were washed with PBS then suspended in cold buffer containing 25 mM Tris, 2 mM EDTA, 1% Triton and protease inhibitor cocktail, and sonicated 2 x 10 seconds using a probe sonicator at low power setting. An aliquot of the homogenate was combined with 20 μl of substrate and sufficient 0.05% BSA, 100 mM potassium phosphate, pH 7.4 buffer to yield 300 μl, then incubated at 37°C for 1 hr. Liposomal substrate, containing cholesteryl-[1-14C]oleate was prepared as described by Kraemer, et al. (36). CE hydrolysis was terminated by add-
ing 50 µl of 2N NaOH. The reaction mixture was extracted using a mixture of methanol/chloroform/benzene (2.4:2:1, v/v), and the \(^{14}\text{C}\) content of the aqueous phase was determined by liquid scintillation counting.

**RESULTS**

**Selection of CETP-deficient clones** - A 549 bp fragment of CETP cDNA was inserted in reverse orientation into pcDNA3, transfected into SW872 adipocytic cells, and stable transfectants selected with Geneticin. After 10-15 days, clones were picked and subcultured under the same selection conditions. Confluent cultures of cells were assayed for CETP expression by determining CETP activity and mass (Western blot) in 48-hr conditioned medium collected in the absence of serum. Among 6 clones, CETP activities ranged 20-100% of control cells. CETP synthesis and secretion were markedly reduced in three of these clones as measured by activity or mass (Figure 1). Comparable reductions in intracellular CETP were also noted for these three clones (Figure 1, inset). Clone 1 was selected for most studies shown. Limited studies are reported for clones 6 and 8.

**Influence of chronic CETP deficiency on CE metabolism** - We previously observed that acute suppression of CETP expression by antisense oligonucleotides caused a small but significant increase in CE following 24 hr of CETP inhibition. Inhibition of CETP expression for three days amplified this effect, resulting in a 2-3 fold increase in CE mass (25). This unusual phenotype, i.e., the accumulation of CE by adipocytes, was exacerbated in cells chronically deficient in CETP. As seen in Figure 2A, the incorporation of \(^{14}\text{C}\)acetate into CE in CETP-deficient cells was 4-fold higher than in control adipocytes. In two other clones expressing similar CETP levels as clone 1, CE was 2.5 fold (clone 6) and 2.0 fold (clone 8) higher than control. We have previously demonstrated in these cells that the CE pool measured using this long-term radiolabel approach mirrors closely that quantitated by direct chemical analysis of CE mass (25). This increased incorporation of \(^{14}\text{C}\)acetate into free fatty acids and into phospholipid was not different between control and CETP-deficient cells (data not shown). Also, the marked increase of \(^{14}\text{C}\)acetate incorporation into CE in CETP-deficient cells was not due to a change in the relative amounts of radioactivity originating from fatty acid versus cholesterol. In control and CETP-deficient cells, 58.7± 2.0 and 62.0± 6.2%, respectively, of \(^{14}\text{C}\) in CE derived from radiolabeled labeled fatty acid.

In cells incubated in 5% LPDS, which promotes cholesterol efflux and reduces CE levels, the CE content of CETP-deficient cells remained >4-fold elevated compared to control cells (Figure 2A). Surprisingly, although exposure of CETP-deficient cells to LPDS reduced their CE content, even after residing in this efflux media for 5 days (2 days pretreatment and 3 days of labeling), these cells still contained more CE than control cells grown under conditions conducive to CE accumulation (10% FBS) (Figure 2A).

To understand the mechanism(s) underlying this increase, we measured the acylation and deacylation activities of the cholesterol cycle. Cells were incubated with \(^{3}\text{H}\)oleate to label the CE pool, then subsequently incubated in media containing HDL to serve as a cholesterol acceptor to promote CE hydrolysis and \(^{14}\text{C}\)oleate to determine the rate of cholesterol reesterification. CETP-deficient cells were aberrant in both CE acylation and deacylation pathways. HDL-stimulated CE hydrolysis in CETP-deficient cells was significantly impaired (Figure 2B). On the other hand, CE synthesis was 2-fold higher in cells with low CETP expression (Figure 2C). The decreased hydrolysis of CE in CETP-deficient cells was not due to a reduction in neutral cholesteryl ester hydrolase, the enzyme that hydrolyzes non-lysosomal CE. Neutral CE hydrolase activity, measured under conditions that approximate enzyme mass (36), was increased more than 3-fold in CETP-deficient cells grown in serum-free media (2.19 ± 0.56 vs. 6.57 ± 1.35 nmoles CE hydrolyzed/mg cell protein/hr) and remained elevated even when cells were grown under conditions (10% FBS) that promote CE accumulation (2.52 ± 0.10 vs. 3.40 ± 0.41 nmoles CE hydrolyzed/mg cell protein/hr, respectively). Further, although extracellular CETP can alter sterol uptake and release by its interaction with media
lipoproteins and/or with the cell surface (21,28,37), these known extracellular functions do not appear to mediate the abnormal CE metabolism in CETP-deficient cells. That is, anti-CETP IgG (>10-fold excess over that needed to block the CETP secreted) did not affect HDL-stimulated CE hydrolysis in control cells, and adding partially purified CETP (equivalent to that secreted by control cells in 48 hr) to the media during the efflux experiment did not restore the rate of CE hydrolysis in CETP-deficient cells (data not shown).

**Cholesterol metabolism in chronically CETP-deficiency cells** - In adipocytes, cholesterol, not CE, is the principal storage sterol. Since sterol metabolism is tightly regulated, and increased CE production is typically associated with suppression of cholesterol biosynthesis, we investigated the status of cholesterol in CETP-deficient cells. Following incubation for 3 days with isotope, [14C]acetate incorporation into cholesterol was low and the same in control and CETP-deficient cells when grown in media containing a source of cholesterol (10% FBS) (Figure 3A). As expected, when control cells were cultured in media that lacked cholesterol and promoted cholesterol removal from cells (5% LPDS), [14C]acetate incorporation into cholesterol was stimulated 5-fold (Figure 3A). This is in marked contrast to CETP-deficient cells where 5% LPDS treatment had little effect on [14C]acetate incorporation. To determine whether the low accumulation of 14C in the cholesterol pool of CETP-deficient cells was due to decreased cholesterol biosynthesis instead of increased conversion to CE, we measured the rate of de novo cholesterol synthesis. The rate of [14C]acetate incorporation into cholesterol was determined in short-term incubations where the incorporation of acetate is linear with time and where little de novo cholesterol is converted to CE. In cells where sterol biosynthesis was upregulated by pre-incubation in media containing LPDS, the cholesterol biosynthetic rate for control cells was 5.2 x 10^4 cpm acetate/mg protein/hr, whereas in CETP-deficient cells this rate was much lower (1.7 x 10^3 cpm acetate/mg protein/hr) (Figure 3B).

The accumulation of CE and down-regulation of cholesterol biosynthesis observed in CETP-deficient cells is typical of cells in cholesterol excess. We subsequently determined whether other aspects of sterol metabolism are consistent with a phenotype of sterol excess. The conversion of cholesterol to CE is mediated by acyl-CoA:cholesterol acyltransferase (ACAT). ACAT activity is driven largely by cholesterol availability (38). ACAT activity in CETP-deficient cells, measured by the incorporation of radiolabeled oleate into CE, was increased 2-fold over control cells (Figure 4A). A phenotype of excess cellular cholesterol is also consistent with the 45% lower LDL receptor activity (Figure 4B) and 30% lower receptor protein levels by western blot (not shown), and the 2.5-fold increase in ABCA1, a cholesterol exporter, observed in CETP-deficient cells (Figures 4C). Collectively, these data show that CETP-deficient cells respond metabolically as if they are cholesterol enriched.

Despite displaying biochemical properties typical of cholesterol-enriched cells, direct determination of cellular cholesterol content gave results contrary to this phenotype. CETP-deficient cells maintained in growth media (10% FBS) contained 50% less cholesterol than control cells (Table 1) even though this media contains abundant cholesterol. When cultured in LPDS-containing media, conditions that stimulate cholesterol efflux and increase cellular cholesterol biosynthesis, CETP-deficient cells remained cholesterol deficient. This may result, in part, from the inability of these cells to upregulate cholesterol biosynthesis appropriately (Figure 3). Further, the lower cholesterol content of CETP-deficient cells was also observed in isolated lipid storage droplets. Droplets isolated from CETP-deficient cells grown in 10% FBS contained 6.6 ± 0.9 versus 10.7 ± 0.1 μg cholesterol/mg protein in control. Together, these data show that although CETP-deficient cells behave metabolically as though they are cholesterol enriched, they are in fact cholesterol poor.

**Influence of chronic CETP deficiency on triglyceride metabolism** - In cells where CETP expression was blunted acutely (24 hr) by antisense oligonucleotides, we observed no alterations in TG metabolism (25). However, since the effect of CETP deficiency on CE accumulation and cholesterol metabolism was exacerbated when CETP suppression was long-term, we investigated whether TG metabolism was altered in chronically CETP-deficient cells. We observed dramatic
changes in adipocyte TG homeostasis. In CETP-deficient cells, TG mass was reduced to ~45% of that in control cells grown in media containing 10% FBS (Table 2). Clones 6 and 8 also showed a similar TG deficiency. When supplemented with oleate, the TG content of both control and CETP-deficient cells increased almost 4-fold, yet CETP-deficient cells remained TG depleted. This deduction in TG mass was easily observed by microscopy (Figure 5A-B). Decreased TG storage resulted in a marked reduction in the number of storage droplets per cell and a reduction in mean storage droplet diameter (1.50 ± 0.016 μm (control) versus 0.99 ± 0.019 μm (CETP-deficient), mean ±S.E.).

Reduced TG content in CETP-deficient cells is at least partially explained by a 50% reduction in the rate of TG biosynthesis, as determined from the incorporation of [3H]oleate (Figure 5C). In separate TG synthesis studies using [14C]acetate, CETP-deficient clones 1 and 6 showed a 37 and 42% reduction in 14C incorporation into TG. Additionally, like that seen for CE, TG in CETP-deficient cells was much less effectively hydrolyzed (Figure 5D). In adipocytes, TG and CE are hydrolyzed by the same enzyme, hormone-sensitive lipase / neutral cholesteryl ester hydrolase (39,40). As shown above, the level of this enzyme is increased in CETP-deficient cells, yet the hydrolysis of TG and CE remains low.

Effect of CETP-deficiency on the cellular distribution of lipids - Both TG and CE are synthesized in the ER, but their hydrolysis in adipocytes is mediated by hormone sensitive lipase / neutral cholesteryl ester hydrolase associated with lipid storage droplets. Although the overall consequences of CETP deficiency on TG and CE levels are distinct, the hydrolysis of both lipids is impaired in CETP-deficient cells. Since CETP can transport both CE and TG, and the efficient hydrolysis of these lipids requires them to be transported to storage droplets, we investigated whether CE and TG are effectively transported in cells with low CETP activity. To examine this possibility, the lipid pools of control and CETP-deficient cells were labeled by incubation with [14C]acetate. Subsequently, cells were homogenized and fractionated by sucrose gradient centrifugation. Under these centrifugation conditions, the lipid droplet marker, perilipin, was mainly recovered in the two uppermost fractions whereas the bulk of the membranes, including ER (as detected by calnexin), were recovered in the bottom 3 fractions. The distribution of lipids in the droplet and ER-containing fractions is shown in Figure 6. For both cell types, the distribution of cholesterol was not different, reflecting primarily the distribution of membranes, especially plasma membrane, in the gradient. In contrast, under these labeling conditions the distribution of CE and TG between cell types was different. For both of these lipids, in control cells ~32% of radiolabel was recovered in the lipid droplet-containing fractions and ~37% was associated with ER-rich fractions. However, in CETP-deficient cells, TG and CE were primarily recovered in the bottom of the gradient. In these cells, <14% of CE and TG were contained in the lipid droplet fraction, and >50% associated with the ER-rich fraction. Like these results found in clone 1, in clone 8 cells only 17% of CE was recovered in lipid droplets. Thus, in CETP-deficient cells, CE and TG, but not cholesterol, are aberrantly distributed among cellular organelles.

Interorganelle transfer of CE and TG by CETP - The abnormal distribution of CE and TG in CETP-deficient cells suggests that CETP may have a direct role in the normal transport of these lipids from their site of synthesis to cellular depots. To test the potential for CETP to mediate such interorganelle transfer, we labeled the lipid pools in CETP-deficient cells, as described in the Experimental Procedures, and then determined the ability of CETP derived from control cells to facilitate the transfer of CE and TG to storage droplets when added to homogenates of these radiolabeled cells. In control experiments, we determined that the addition of 300 μM diethylumbeliferyl phosphate was required to suppress the hydrolysis of CE and TG by endogenous lipases and stabilize CE and TG levels during the transfer experiment. When cytosol from CETP-deficient cells, which contained low CETP by western blot (not shown) was added to the radiolabeled homogenate, ~10 and 5% of CE and TG, respectively, was recovered in the droplet fraction after 8 hr (Figure 7). This distribution was not consistently different from that present in homogenates at time zero (not shown). However, when cytosol from control cells containing native levels of CETP was added there was a marked 2-
3-fold increase in the amount of CE and TG associated with lipid droplets (Figure 7). This rise in droplet-associated CE and TG was quantitatively accounted for by the loss of radioactivity in the ER-rich fraction (not shown). Preincubation of control cell cytosol with a CETP monoclonal antibody prevented this redistribution of CE and TG (Figure 7), demonstrating that CETP is responsible for this interorganelle transfer.

DISCUSSION

In this study we examined the effect of chronic, partial CETP deficiency on lipid metabolism in an adipocyte model cell line. Long-term CETP suppression exacerbated the alterations in lipid metabolism previously noted during short-term CETP suppression (25), and elicited other aberrations. In chronically deficient cells, cellular CE increased ~4 fold, which resulted from both increased CE synthesis and decreased degradation. Further, extended CETP suppression decreased both the synthesis and degradation rates for TG. The deficiencies in CE and TG hydrolysis were not accounted for by low levels of the lipase that cleaves both of these lipids in adipocytes (39,41,42), suggesting that these lipid substrates may not be appropriately presented to the lipase in CETP-deficient cells. Consistent with this hypothesis, we observed in CETP-deficient cells that recently synthesized CE and TG are ineffectively transported to storage droplets where the functional lipase resides. In an in vitro assay we directly demonstrated that cellular CETP could mediate the transport of CE and TG from ER-rich membranes, where they are biosynthesized, to storage droplets. Collectively, these findings are consistent with the conclusion that CETP deficiency causes CE and TG to accumulate in the ER, resulting in aberrant lipid storage and disruption of lipid metabolic pathways.

Given the primary role of adipocytes as TG storage depots, perhaps the most striking consequence of partial CETP deficiency in SW872 cells is a 50% reduction in the amount of TG that is stored in these cells. CETP-deficient cells visibly contain fewer and smaller TG-rich storage droplets. Based on acetate incorporation, CETP-deficient cells have normal fatty acid synthetic capacity and the incorporation of fatty acids into complex lipids such as phospholipids is unaffected. This suggests that the low TG content, which is mirrored by a reduction in TG synthetic capacity, may be due to aberrant regulation of diacylglycerol acyltransferase.

In addition to abnormal TG and CE metabolism, cholesterol metabolism was altered in CETP-deficient cells. Although cholesterol synthesis in mature adipocytes is normally down regulated, it can be robust when the uptake of exogenous cholesterol sources is compromised, such as in homozygous familial hypercholesterolemia (43), or when cellular lipid content is low (43,44). This appears to be the case in control SW872 adipocytes. However, in CETP-deficient cells we observed that the rate of cholesterol biosynthesis was significantly reduced and that these cells were unable to appropriately upregulate sterol biosynthesis in response to stimuli. This decreased cholesterol synthesis was accompanied by down regulation of LDL receptor activity and upregulation of ABCA1 protein levels. Together, these responses, which are likely mediated by LXR- and SREBP-linked, sterol-dependent mechanisms (45), typify cells in cholesterol excess. However, direct chemical analysis of CETP-deficient cells showed them to be cholesterol deficient. These data strongly suggest that CETP-deficiency disrupts sterol homeostasis by inducing erroneous sensing of the cell’s sterol status, perhaps through perturbation of regulatory sterol pools in the ER.

In contrast to other cells, cholesterol stored in adipocytes is >90% in the free form. However, cholesterol in adipocytes has a slow turnover, and membrane cholesterol and droplet cholesterol pools are metabolically distinct as exemplified by the fact that these pools vary inversely during adipocyte maturation (44,46). Thus, it is not likely that stored cholesterol derives directly from the membrane pool by equilibration. In fact, a priori, there is no reason to believe that the mechanism of cholesterol storage in adipocytes differs from that in other cells. That is, excess cholesterol, which is deleterious to cells (47), is converted to CE and stored in cytoplasmic droplets. CE in storage droplets continuously undergoes hydrolysis to cholesterol and, if the cholesterol is not needed, it is transported back to the ER for reesterification and then redeposit in the droplet (48,49). In the adipocyte, we propose that the product of CE hydrolysis, cholesterol, does not readily leave the
droplet because of its solubility in TG (50,51), leading to a steady state where the bulk of cholesterol in the adipocyte droplet is in the free form. This is supported by the finding that the loss of cholesterol from adipocyte droplets is minimal during the first 24 hr of stimulated TG lipolysis (52,53) but increases thereafter, suggesting that cholesterol is not released from storage droplets until the solubility limit in the TG phase is approached. Likewise, when TG accumulation is blocked, adipocytes fail to accumulate cholesterol (54).

In view of the foregoing observations, we propose that the down-regulation of sterol synthesis, the inappropriate accumulation of CE in ER-rich membranes, and the low level of cholesterol present in storage droplets of CETP-deficient cells may have a common link. We hypothesize that CETP-deficiency impairs the transport of CE from its site of synthesis to the storage droplet. This in turn lowers the cholesterol content of storage droplets since it is derived from the hydrolysis of CE. Finally, the inefficient removal of sterols from the ER leads to a signal of sterol abundance, causing down regulation of pathways that elevate cholesterol levels (LDL receptor, de novo biosynthesis), and upregulation of cholesterol clearance pathways (ABCA1). While the lower TG levels in CETP-deficient cells may contribute to the lower cholesterol content by a mechanism such as that mentioned above, this appears unlikely to be a major or precipitating cause since defects in CE and cholesterol metabolism temporally precede changes in TG metabolism induced by CETP inhibition (25).

Overall, we interpret these data to show that partial CETP deficiency causes the ectopic accumulation of CE and TG at their site of synthesis instead of being transported to storage droplets, and that this erroneous deposition of CE and TG perturbs lipid metabolic pathways and places these lipids in a cellular location where they are poorly accessible to hydrolytic enzymes. These data suggest that CETP, which has a well known role in interlipoprotein transport of CE and TG, may have a similar role intracellularly, as has been recently supported by other studies (23-25). How CETP may perform such a function is unclear. CETP has broad substrate specificity in vitro, promoting lipid transfers among liposomes, lipoproteins and between membranes, including rough and smooth ER (18,19). So CETP may facilitate cytoplasmic interorganelle transfer as we demonstrated in vitro, and/or it may have a function analogous to that of microsomal triglyceride transfer protein (MTP). MTP is essential for the transport of TG from the ER membrane into the ER lumen where TG-rich droplets are formed; these droplets then fuse with nascent apolipoprotein B particles to yield mature lipoproteins (55,56). Similarly, luminal CETP may concentrate CE and TG into specialized regions of the ER where nascent storage droplets form and bud into the cytoplasmic space (57). Since our antisense strategy blocks the production of both full-length and exon-9 deleted CETP, our study does not address which forms of CETP mediate these intracellular functions. Regardless of the mechanism ultimately identified, our data provide strong support for the conclusion that CETP is essential for normal lipid metabolism and storage in adipocytes. This is consistent with the recent observation in mice that adipose tissue-specific CETP expression alters adipocytes size and their content of TG and cholesterol (58). It follows that CETP-deficient humans may have abnormalities in adipose tissue function. Studies directly examining this interesting possibility have not been reported.

The newly discovered secretory functions of adipocytes have shifted the view of adipose tissue from being a simple energy storage tissue to one where this tissue functions as a major endocrine organ. In addition to their cholesterol and TG storage function, adipocytes also synthesize and secrete a variety of factors such as leptin, adiponectin, angiotensinogen, resistin and lipoprotein lipase, that regulate whole body energy balance and lipid homeostasis (59,60). The secretion of these factors is closely linked to the lipid status of adipocytes. Both hypertrophy (excess of lipid content) and hypotrophy (low lipid content) of adipocytes have been shown to disrupt the secretion of these factors and cause abnormal whole body metabolism and inadequate insulin responsiveness (59,60). Our studies demonstrate that CETP deficiency leads to abnormal TG and cholesterol storage and lowers the membrane ratio of FC/protein, factors reported to be associated with induction of insulin resistance and alteration in the synthesis of adipocytokines (44). Our find-
ings, if they can be extrapolated to adipose tissue, suggest an important role for CETP in regulating the multiple functions of adipocytes.

REFERENCES

1. Pattnaik, N. M., Montes, A., Hughes, L. B., and Zilversmit, D. B. (1978) *Biochim. Biophys. Acta* **530**, 428-438
2. Morton, R. E., and Zilversmit, D. B. (1982) *J. Lipid Res.* **23**, 1058-1067
3. Hesler, C. B., Swenson, T. L., and Tall, A. R. (1987) *J. Biol. Chem.* **262**, 2275-2282
4. Hesler, C. B., Tall, A. R., Swenson, T. L., Weech, P. K., Marcel, Y. L., and Milne, R. W. (1988) *J. Biol. Chem.* **263**, 5020-5023
5. Bruce, C., Chouinard, R. A., Jr., and Tall, A. R. (1998) *Annu. Rev. Nutr.* **18**, 297-330
6. Bruce, C., Beamer, L. J., and Tall, A. R. (1998) *Curr. Opin. Struct. Biol.* **8**(4), 426-434
7. Inazu, A., Quinet, E. M., Wang, S., Brown, M. L., Stevenson, S., Barr, M. L., Moulin, P., and Tall, A. R. (1992) *Biochemistry* **31**, 2352-2358
8. Yang, T. P., Agellon, L. B., Walsh, A., Breslow, J. L., and Tall, A. R. (1996) *J. Biol. Chem.* **271**(21), 12603-12609
9. Wang, S., Deng, L., Milne, R. W., and Tall, A. R. (1992) *J. Biol. Chem.* **267**, 17487-17490
10. Wang, S., Kussie, P., Deng, L., and Tall, A. (1995) *J. Biol. Chem.* **270**(2), 612-618
11. Roy, P., MacKenzie, R., Hirama, T., Jiang, X. C., Kussie, P., Tall, A., Rassart, E., and Milne, R. (1996) *J. Lipid Res.* **37**(1), 22-34
12. Jiang, X., Bruce, C., Cocke, T., Wang, S., Boguski, M., and Tall, A. R. (1995) *Biochemistry* **34**(21), 7258-7263
13. Drayna, D., Jamagin, A. S., McLean, J., Henzel, W., Kohr, W., Fielding, C., and Lawn, R. (1987) *Nature* **327**, 632-634
14. Jiang, X. C., Moulin, P., Quinet, E., Goldberg, I. J., Yacoub, L. K., Agellon, L. B., Compton, D., Schnitzer-Polokoff, R., and Tall, A. R. (1991) *J. Biol. Chem.* **266**, 4631-4639
15. Albers, J. J., Tollefson, J. H., Wolfbauer, G., and Albright, R. E., Jr. (1992) *Int. J. Clin. Lab. Res.* **21**, 264-266
16. Faust, R. A., Tollefson, J. H., Chait, A., and Albers, J. J. (1990) *Biochim. Biophys. Acta* **1042**, 404-409
17. Zhang, Z., Yamashita, S., Hirano, K., Nakagawa-Toyama, Y., Matsuyama, A., Nishida, M., Sakai, N., Fukasawa, M., Arai, H., Miyagawa, J., and Matsuzawa, Y. (2001) *Atherosclerosis* **159**(1), 67-75
18. Morton, R. E. (1990) *Experientia* **46**, 552-560
19. Hashimoto, S., Morton, R. E., and Zilversmit, D. B. (1984) *Biochem. Biophys. Res. Commun.* **120**, 586-592
20. Pattnaik, N. M., and Zilversmit, D. B. (1979) *J. Biol. Chem.* **254**, 2782-2786
21. Benoist, F., Lau, P., McDonnell, M., Doelle, H., Milne, R., and McPherson, R. (1997) *J. Biol. Chem.* **272**(38), 23572-23577
22. Gauthier, A., Lau, P., Zha, X., Milne, R., and McPherson, R. (2005) *Arterioscler. Thromb. Vasc. Biol.* **25**(10), 2177-2184
23. Sawada, S., Sugano, M., Makino, N., Okamoto, H., and Tsuchida, K. (1999) *Atherosclerosis* **146**(2), 291-298
24. Huang, Z., Inazu, A., Kawashiri, M. A., Nohara, A., Higashikata, T., and Mabuchi, H. (2003) *Am. J. Physiol. Endocrinol. Metab.* **284**, E1210-E1219
25. Izem, L., and Morton, R. E. (2001) *J. Biol. Chem.* **276**(28), 26534-26541
26. Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955) *J. Clin. Invest.* **34**, 1345-1353
27. Morton, R. E., and Zilversmit, D. B. (1983) *J. Biol. Chem.* **258**, 11751-11757
28. Morton, R. E. (1988) *J. Lipid Res.* **29**, 1367-1377
29. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Phys.* **37**, 911-917
30. Gamble, W., Vaughan, M., Kruth, H. S., and Avigan, J. (1978) *J. Lipid Res.* **19**, 1068-1070
31. Mendez, A. J., Cabeza, C., and Hsia, S. L. (1986) *Anal. Biochem.* **156**, 386-389
32. Brasaemle, D. L., Rubin, B., Harten, I. A., Gruia-Gray, J., Kimmel, A. R., and Londos, C. (2000) *J. Biol. Chem.* **275**(49), 38486-38493
33. Goldstein, J. L., and Brown, M. S. (1974) *J. Biol. Chem.* **249**, 5153-5162
34. MacFarlane, A. S. (1958) *Nature* **182**, 53-57
35. Bilheimer, D. W., Eisenberg, S., and Levy, R. I. (1972) *Biochim. Biophys. Acta* **260**, 212-221
36. Kraemer, F. B., Patel, S., Saedi, M. S., and Szalayrd, C. (1993) *J. Lipid Res.* **34**, 663-671
37. Clay, M. A., Hopkings, G. J., Elnholm, C. P., and Barter, P. J. (1989) *Biochim. Biophys. Acta* **1002**, 173-181
38. Chang, T. Y., Chang, C. C. Y., and Cheng, D. (1997) *Annu. Rev. Biochem.* **66**, 613-638
39. Hajjar, D. P. (1994) *Adv. Enzymol. Relat. Areas Mol. Biol.* **69**, 45-82
40. Large, V., Anier, P., Reynisdottir, S., Grober, J., Van Harmelen, V., Holm, C., and Langin, D. (1998) *J. Lipid Res.* **39**, 1688-1695
41. Fredrickson, G., Striflers, P., Nilsson, N. O., and Belfrage, P. (1981) *J. Biol. Chem.* **256**, 10583-10588
42. Kraemer, F. B., and Shen, W. J. (2002) *J. Lipid Res.* **43**, 1585-1594
43. Krause, B. R., and Hartman, A. D. (1984) *J. Lipid Res.* **25**, 97-110
44. Le Lay, S., Krief, S., Farnier, C., Lefrere, I., Le Liepvre, X., Bazin, R., Ferré, P., and Dugail, I. (2001) *J. Biol. Chem.* **276**, 16904-16910
45. Edwards, P. A., Kast, H. R., and Anisfeld, A. M. (2002) *J. Lipid Res.* **43**, 2-12
46. Le Lay, S., Robichon, C., Le Liepvre, X., Dagher, G., Ferre, P., and Dugail, I. (2003) *J. Lipid Res.* **44**(8), 1499-1507
47. Tabas, I. (2002) *J. Clin. Invest.* **110**, 905-911
48. Brown, M. S., and Goldstein, J. L. (1983) *Ann. Review Biochem.* **52**, 223-261
49. Kellner-Weibel, G., McHendry-Rindle, B., Haynes, M. P., and Adelman, S. (2001) *J. Lipid Res.* **42**, 768-777
50. Miller, K. W., and Small, D. M. (1983) *Biochemistry* **22**, 443-451
51. Miller, K. W., and Small, D. M. (1983) *J. Biol. Chem.* **258**, 13772-13784
52. Dugail, I., Le Lay, S., Varret, M., Le Liepvre, X., Dagher, G., and Ferreé, P. (2003) *Horm. Metab. Res.* **35**, 204-210
53. Krause, B. R., Balzer, M., and Hartman, A. D. (1981) *Proc. Soc. Exp. Biol. Med.* **167**, 407-411
54. Zechner, R., Moser, R., Newman, T. C., Fried, S. K., and Breslow, J. L. (1991) *J. Biol. Chem.* **266**, 10583-10588
55. Gordon, D. A., and Jamil, H. (2000) *Biochim. Biophys. Acta* **1486**, 72-83
56. Kulinski, A., Rustaeus, S., and Vance, J. E. (2002) *J. Biol. Chem.* **277**(35), 31516-31525
57. Murphy, D. J., and Vance, J. (1999) *Trends Biochem. Sci.* **24**, 109-115
58. Zhou, H., Li, Z., Hojati, M. R., Jang, D., Beyer, T. P., Cao, G., Tall, A. R., and Jiang, X. C. (2006) *Journal of Lipid Research* **47**(9), 2011-2019
59. Mora, S., and Pessin, J. E. (2002) *Diabetes Metab. Res. Rev.* **18**, 345-356
60. Walczak, R., and Tontonoz, P. (2002) *J. Lipid Res.* **43**, 177-186

FOOTNOTES

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1The abbreviations used are: CETP, cholesteryl ester transfer protein; LDL, low density lipoprotein; HDL, high density lipoprotein; CE, cholesteryl ester, TG, triglyceride; LPDS, lipoprotein deficient serum;
DMEM/F-12, Dulbecco modified Eagle’s medium/Ham’s F-12 medium; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ACAT, acylCoA:cholesterol acyltransferase; ER, endoplasmic reticulum.

FIGURE LEGENDS

**Figure 1:** CETP levels in control cells and stable antisense clones. Control SW872 cells and select clones stably expressing CETP antisense cDNA were grown to near confluence in 100 mm dishes, then incubated in 5 ml serum free media for 48hr. Conditioned media was collected and assayed for CETP activity. The activities measured in 300 µl of control media, and comparable volumes of clone media, normalized by the cell count of each dish, are shown. Values are the mean ± S.D. of triplicate determinations. CETP in conditioned media and in cell homogenates was immunoprecipitated and subjected to western blot (inset). PAGE gels received the immunoprecipitated protein from 400 µl control media, and comparable volumes of clone media (normalized for differences in cell count), or the protein immunoprecipitated from 200 µg of cell homogenates. See Experimental Procedures for details.

**Figure 2:** Effect of chronic CETP-deficiency on cholesteryl ester metabolism. A – CE synthesis in SW872 cells. Confluent cells were pretreated with media ± 5% LPDS overnight then incubated either in media containing 5% LPDS or 10% FBS and [14C]-acetate for 4 days. The cells were washed extensively and the lipid were extracted and analyzed. Values are the mean ± S.D. of triplicates. The rates of CE hydrolysis (B) and CE synthesis (C) were measured by incubating the cells in DMEM/F-12 containing 1% BSA and 100 µM [3H]oleate/BSA (1.2 x 10^4 cpm/nmole). After 48 hr, cells were washed, then serum-free media containing 1% BSA, 100 µM [14C]oleate/BSA (3.5 x 10^3 cpm/nmole) and 100 µg HDL/ml was added. Cells were assayed for [3H]- and [14C]-CE content at the indicated times. See Experimental Procedures for details. Results are typical of 3 similar experiments. All control versus CETP-def. (CETP-deficient) comparisons were significantly different (p ≤ 0.03) except for the 12 hr point in panel B.

**Figure 3:** Cholesterol metabolism in chronic CETP-deficiency. A – Free cholesterol content. Confluent cells were pretreated in media ± 5% LPDS overnight then incubated either in media containing 5% LPDS or 10% FBS and [14C]-acetate for 4 days. The cells were washed extensively and the lipids were extracted and analyzed. B – Rate of cholesterol synthesis. SW872 adipocytes were pretreated as in Panel A, then incubated in media containing 5% LPDS and [14C]acetate for the indicated times. [14C]acetate incorporation into cholesterol was determined as described in the Experimental Procedures. All control versus CETP-def. (CETP-deficient) comparisons were significantly different (p ≤ 0.001) except for the FBS condition in Panel A. Values are the mean ± S.D of triplicates, and are representative of 2-3 experiments.

**Figure 4:** CETP deficiency alters sterol-responsive proteins. A - ACAT activity. Cell were grown to near confluence in media containing 10% FBS, then incubated in serum-containing media supplemented with 500 µM [3H]oleate/BSA. After 5 hr, cells were harvested and [3H]CE was determined. Values are mean ± S.D. of triplicates. B – LDL uptake. Cell were treated with media containing 5% LPDS overnight then incubated in the same media containing 50 µg/ml [125I]LDL for 5 hr at 37°C. The cells were washed extensively and the amounts of the label present in the cells and degraded in the media were determined as described in Experimental Procedures. Values are the mean ± S.D. (n=4) and representative of 3 similar experiments. C – Western blot showing ABCA1 expression level. Cell proteins (50 µg protein) were separated on 7.5% SDS-PAGE and transferred to a PVDF membrane. ABCA1 was detected using a polyclonal antibody against human ABCA1 and goat anti-rabbit IgG coupled with peroxidase. RAW mouse macrophages are a positive control showing c-AMP mediated induction of ABCA1 expression.

**Figure 5:** Effect of chronic CETP deficiency on TG metabolism. A – Oil Red O staining in control SW872 cells. Cells were incubated in media containing 250 µM oleate for 48 hr. Fixed cells were stained with oil red O and DAPI as indicated in Experimental Procedures. Shown are confocal images near the mid-point of the cell. B - Oil Red O staining in CETP-deficient cells. See Panel A description above for
details. C - Rate of TG synthesis. Confluent cells were incubated in serum-containing media supplemented with 100 μM [3H]oleate/BSA for the indicated times. Cells were washed, and radiolabeled TG was determined. See Experimental Procedures for details. D - Rate of TG hydrolysis. TG hydrolysis was determined by measuring the loss of radiolabeled TG, synthesized by preincubation of cells in [3H]oleate/BSA, in the presence Triacsin D to block further TG synthesis. See Experimental Procedures for details. Values in Panels C and D are the mean ± S.D. of triplicates, and are representative of 3 similar experiments.

**Figure 6:** Effect of chronic CETP deficiency on free cholesterol, CE and TG distribution in SW872 adipocytes. Cells at 100% confluence were incubated in 10% FBS-containing media supplemented with [14C]acetate for 3 days. The cells were washed extensively, homogenized, and fractionated on a sucrose gradient as described in the Experimental Procedures. Ten fractions were collected from the gradient and analyzed for radiolabeled cholesterol, CE and TG content. Based on perilipin (lipid storage droplets) and calnexin (endoplasmic reticulum, ER) distribution, data from the top 2 and bottom 3 fractions were combined to yield values for “droplet” and “ER rich” fractions, respectively. In both cell types, the remaining radioactive was equally distributed among the 5 intervening fractions. These results are representative of 3 similar experiments.

**Figure 7:** Interorganelle transfer of CE and TG by cellular CETP. CETP-deficient cells were incubated with [3H]oleate as indicated in Experimental Procedures to generate organelle-associated radiolabeled CE and TG. Following homogenization of these cells, aliquots (1 mg protein) were combined with cytosol from unlabeled CETP-deficient or control cells (300 μg protein) and incubated at 37°C for 8 hr, as indicated. When indicated, TP2 (8μg), a monoclonal antibody against CETP, was preincubated with control cytosol (1 hr, 37°C) prior to addition the transfer assay. Subsequently, samples were fractioned by sucrose gradient centrifugation. Shown are the percentages of total CE or TG that were recovered in the top three fractions of the gradient (storage droplet fraction). Values are mean ± S.D. (n=3). These results are representative of 3 experiments.
TABLE 1

Cholesterol Content of Control and CETP-deficient Cells. Control and CETP-deficient cells were grown in media containing 5% serum or 10% LPDS for 2 days, washed extensively, harvested, and assayed for their cholesterol content by a fluorometric, enzymatic assay. Values are the mean ± S.D of triplicates, and representative of 3 experiments.

| Culture condition | Control | CETP-deficient |
|-------------------|---------|----------------|
| FBS               | 42.9 ± 1.3 | 21.7 ± 0.4<sup>a</sup> |
| LPDS              | 35.1 ± 3.9 | 19.9 ± 0.5<sup>b</sup> |

Unpaired t test comparison of control and CETP-deficient cells: <sup>a</sup>P< 0.0001; <sup>b</sup>P= 0.0026.

TABLE 2

Triglyceride Content of Control and CETP-deficient Cells. Control SW872 adipocytes and three unique clones of SW872 cells deficient in CETP (CETP-def.) were grown in media containing 10% FBS with or without 500 μM oleic acid/BSA for 48 hr. Cells were washed extensively, harvested, and TG mass was determined by fluorometric assay. All control versus CETP-def. (CETP-deficient) comparisons were significantly different (p< 0.01). Values are the mean ± S.D. (n = 4), and are representative of 2-3 similar experiments. n.d. - not determined.

| Cell type           | FBS       | FBS + Oleate |
|---------------------|-----------|--------------|
| Control             | 24.4 ± 2.4| 98.3 ± 12.3  |
| CETP-def., clone 1  | 11.3 ± 0.6| 41.3 ± 3.3   |
| CETP-def., clone 6  | 13.1 ± 1.2| 38.5 ± 0.5   |
| CETP-def., clone 8  | 11.1 ± 0.2| n.d.         |
Figure 1

[Graph showing CETP activity (% transfer) for different cell types and clones. Bars represent the media and cell CETP activity for Control, Clone 1, Clone 6, and Clone 8.]
Figure 2

(A) CE content (cpm/mg protein) x 10^{-4}

Treatment: FBS, LPDS
Control, CETP-def.

(B) CE hydrolyzed (% control)

Time (hr): 0, 10, 20, 30, 40
Control, CETP-def.

(C) CE synthesis (cpm) x 10^{-2}

Time (hr): 0, 10, 20, 30, 40
Control, CETP-def.
Figure 3

(A) Cholesterol (cpm/mg protein) x 10^{-5} for FBS and LPDS treatments, showing higher cholesterol synthesis in LPDS compared to FBS.

(B) Cholesterol synthesis (cpm/mg protein) x 10^{-4} over time (min), with CETP-def. and Control treatments, showing increased synthesis over time for both groups.
Figure 4

A) ACAT activity (nmol oleate/mg protein)

B) LDL uptake (µg/mg cell protein/5hr)

C) Western blot

Control CETP-def. SW872
Control CETP-def.

Cell type

p<0.002

p<0.001

Anti-ABCA1
Figure 5

A and B: Images showing the synthesis and degradation of TGs.

C and D: Graphs showing the time course of TG synthesis (C) and degradation (D) in control and CETP-deficient conditions.
Figure 6

Lipid distribution (% of cell total)

- **FC**: Full Circles
- **CE**: Crosses
- **TG**: Stripes

**Cell Fraction**

- **Control**
- **CETP-deficient**

**Lipid Distribution**

- **droplet**
- **ER-rich**
Figure 7

CETP-def. Control Control + TP2

CE or TG in storage droplet (% of total in cell)

Cytosol added to assay

CE

TG
Possible role for intracellular cholesteryl ester transfer protein in adipocyte lipid metabolism and storage
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