Cholesteryl ester transfer protein biosynthesis and cellular cholesterol homeostasis are tightly interconnected

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SUMMARY

Cholesteryl ester transfer protein (CETP) mediates triglyceride (TG) and cholesteryl ester (CE) transfer between lipoproteins, and its activity is strongly modulated by dietary cholesterol. To better understand the regulation of CETP synthesis, and the relationship between CETP levels and cellular lipid metabolism, we selected the SW872 adipocytic cell line as a model. These cells secrete CETP in a time-dependent manner at levels exceeding those observed for Caco-2 or HepG2 cells. The addition of LDL, 25OH-cholesterol, oleic acid or acetylated LDL to SW872 cells increased CETP secretion (activity and mass) up to 6-fold. In contrast, CETP production was decreased by almost 60% after treatment with lipoprotein-deficient serum or β-cyclodextrin. These effects, which were paralleled by changes in CETP mRNA, show that CETP biosynthesis in SW872 cells directly correlates with cellular lipid status. To investigate a possible, reciprocal relationship between CETP expression and cellular lipid homeostasis, CETP biosynthesis in SW872 cells was suppressed with CETP antisense oligonucleotides. Antisense oligonucleotides reduced CETP secretion (activity and mass) by 60% compared to sense-treated cells. When CETP synthesis was suppressed for 24 hr, TG synthesis was unchanged but cholesterol biosynthesis was reduced by 20% and acetate incorporation into CE increased 31%. After 3 days of suppressed CETP synthesis, acetate incorporation into the CE pool increased 3-fold over control. This mirrored a similar increase in CE mass. The efflux of free cholesterol to HDL was the same in sense and antisense-treated cells; however, HDL-induced CE hydrolysis in antisense-treated cells was diminished 2-fold even though neutral CE hydrolase activity was unchanged. Thus, CETP-compromised SW872 cells display a phenotype characterized by inefficient mobilization of CE stores leading to CE accumulation. These results strongly suggest that CETP expression levels contribute to normal cholesterol homeostasis in adipocytic cells. Overall, these studies demonstrate that lipid homeostasis and CETP expression are tightly coupled.
INTRODUCTION

Cholesteryl ester transfer protein (CETP)\(^1\) is a plasma glycoprotein that mediates the transfer of neutral lipids between lipoproteins (1,2). Plasma CETP levels are influenced by dietary cholesterol, hyperlipidemia, hormones, and drugs (3,4) and its activity is modulated by CETP mass, lipoprotein levels, and by a circulating inhibitor (5). CETP mRNA is expressed in a number of tissues (6). In humans, liver, spleen and adipose tissue are the most abundant sources of CETP mRNA (7). Studies in non-human primates also demonstrate that adipose tissue expresses high levels of CETP mRNA (8,9). All human tissues expressing CETP contain both a full-length form, which gives rise to plasma CETP, as well as a shortened mRNA in which the exon 9-derived sequence has been deleted (10). The product of this truncated message is poorly secreted, but retains all sequences known to be necessary for lipid transfer activity (11).

Growing evidence indicates that CETP significantly modulates lipoprotein metabolism, including the multi-step process known as reverse cholesterol transport. Genetic alterations in CETP levels, in humans and transgenic mice, are associated with impairment of important steps involved in reverse cholesterol transport (12). Additionally, elevated CETP levels increase the rate at which cholesteryl ester (CE) returns to the liver (13,14). While some of these effects are mediated through the actions of circulating CETP, the widespread tissue distribution of CETP mRNA raises the possibility that CETP synthesized by various peripheral tissues may have local functions in lipid metabolism as well. Such a dual role would help explain why some species that do not have circulating CETP have a CETP-like gene and express detectable CETP message in various tissues (4). Indeed, it has been shown that CETP enhances sperm capacitation (15) and facilitates the efflux of CE from cells (16). Additionally, CETP associates with cell plasma membranes where it appears to facilitate CE selective uptake (17).

While the regulation of CETP activity in plasma, and its responsiveness to dietary cholesterol, has been extensively studied, the molecular mechanisms involved in regulating CETP expression have been difficult to dissect. This is at least partly due to the lack of reproducible cell models where these regulatory events can be studied most easily. Although several cell lines have been reported to synthesize and secrete CETP (18-21), it remains to be determined whether these cultured cells regulate CETP biosynthesis in a physiologically relevant manner and how this regulation is integrated with cellular lipid homeostasis. Given the reported secretion of CETP by the SW872 adipocytic cell line (21), and the physiological
importance of adipose tissue in CETP biosynthesis (22), we have investigated the regulation of CETP expression in this human liposarcoma. We report here that CETP synthesis in SW872 cells is closely correlated with cellular lipid status, and that CETP synthesis responds to lipid stimuli in a manner analogous to that seen in vivo. We also show for the first time that cellular lipid homeostasis in the SW872 cell line is dependent on the normal expression of CETP.
EXPERIMENTAL PROCEDURES

Material - The human colon adenocarcinoma Caco-2 (ATCC# HTB-37), the human liposarcoma cell line SW872 (ATCC# HTB-92), and the hepatocarcinoma HepG2 (ATCC# HB-8065) were purchased from American Type Culture Collection (ATCC, Rockville, MD). Dulbecco Modified Eagle’s/ Ham’s F12 Medium (DMEM/F12) was obtained from Gibco BRL (Grand Island, NY), and fetal bovine serum was from Bio Whitaker. LDL, HDL, and other lipoproteins were isolated from fresh human plasma as described (23). Acetylated LDL was prepared by repetitive additions of acetic anhydride (24). Penicillin, streptomycin, bovine serum albumin, sodium oleate, and β-cyclodextrin were from Sigma (St. Louis, MO). 3H-cholesterol (1,2- 3H(n), 43.5 Ci/mmol), 3H-oleic acid (9, 10- 3H(n), 5.0 Ci/mmol), and 14C-acetic acid sodium salt (1- 14C, 55.0 mCi/mmol) were from New England Nuclear (Boston, MA). Guinea pig anti-perilipin antibody and donkey anti-guinea IgG peroxidase conjugate were from Research Diagnostics, Inc., (Flander, NJ).

Immobilized protein A was from Pierce (Rockford, Illinois). The human CETP cDNA (CETP.11 ATCC # 59792) was purchase from ATCC.

Cell culture - All cells (HepG2, SW872 and Caco-2) were cultured in DMEM/F12 media (3:1) containing 10% fetal bovine serum, and 50 µg/ml penicillin/streptomycin, in 5% CO2/ 95% air at 37°C. For experiments, Caco-2 cells were cultured on transwell filters (Corning Costar Corporation, Cambridge, MA). When cells achieved 100% confluence, the spent medium was aspirated and fresh DMEM/F12 media was added. Conditioned media, collected at the indicated times, was centrifuged briefly to remove cell debris, then assayed for CE transfer activity to determine basal secretion rates. For Caco-2 cells, transfer activity was measured in the lower (basolateral) compartment (20).

Effect of cellular lipid status on CETP secretion - To assess the effects of various lipid donors on CETP synthesis and secretion, cells were pre-treated overnight with DMEM/F12 media containing 5% LPDS. Subsequently, cells were washed thoroughly with serum-free media, and then incubated for 24 hr with DMEM/F12 containing native LDL (100 µg/ml), oleate-BSA (500 µM), acetylated LDL (100 µg/ml) or 25-OH-cholesterol (100 µM). In experiments where the effect of lipid depletion on CETP secretion was studied, cells were cultured in serum-containing media (10% FBS) overnight, washed thoroughly, then incubated with DMEM/F12 media containing 5% LPDS, 500 µM cyclodextrin (25) or both LPDS and β-
cyclodextrin for 24 hr. After either protocol, the treatment medium was removed, cells were washed, then incubated in DMEM/F12 media alone for 48 hr. CETP activity and mass was measured in conditioned media as described below. Cell protein content was measured by the method of Lowry (26, 27).

To determine the influence of TG content on CETP secretion, SW872 cells were pre-treated with DMEM/F12 containing 5% LPDS for overnight then incubated with DMEM/F12 supplemented with 200 µM sodium oleate/BSA (1 to 4 days). At the indicated times, this medium was replaced by DMEM/F12 alone and incubated for an additional 48 hr. Conditioned media was assayed for CE transfer activity and the TG mass content of cells was measured enzymatically (28).

**Cholesteryl ester transfer assay** - Cholesteryl ester transfer assays were carried out as described (29). Briefly, radiolabeled donor lipoprotein (H-LDL, 10 µg cholesterol) and unlabeled acceptor lipoprotein (HDL, 10 µg cholesterol) were incubated with conditioned media (100-200 µl) at 37°C for 18 hr. The assay was terminated by selectively precipitating LDL (donor) by addition of sodium phosphate and MnCl₂. The extent of radiolabeled CE transferred to HDL (supernatant) was calculated as previously described (29). In some instances, samples were preincubated for 30 minutes with 10 µg of anti-CETP IgG (TP2) (30) before initiating the transfer assay.

**Western blotting of CETP in conditioned media** - CETP protein secreted into the media was determined by western blot analysis. Briefly, 5 to 6 ml of conditioned media was concentrated to 1 ml using Centriprep-10 concentrators (Millipore Corp.) and incubated with immunoprecipitation buffer (200 mM NaH₂PO₄, pH 7.5, 500 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 0.02% sodium azide) containing a polyclonal antibody (1:1000) raised against human CETP (16). After overnight incubation at 4°C, 10 µl of immobilized protein A was added and incubated for 2 hr, and the protein/agarose complex was pelleted by centrifugation and washed extensively. CETP was eluted from the agarose by adding 50 µl of 1 M glycine, pH 2.5, combined with gel-loading buffer, boiled for 5 min, and subjected to 7.5% SDS-polyacrylamide gel electrophoresis. Western blot was accomplished using TP2 anti-CETP antibody (30) and anti-mouse IgG coupled with horseradish peroxidase.

**Ribonuclease protection assay** - Cells at 100% confluence were pretreated with medium containing 5% LPDS for 24 hr then treated with different lipids for an additional 24 hr. Total RNA was then isolated.
using trizol reagent according to the manufacturer’s protocol (Gibco BRL). CETP mRNA levels were analyzed by ribonuclease protection assay using the Ambion RPAIII kit (Ambion) and an antisense RNA probe. The antisense CETP riboprobe was prepared using T7 RNA polymerase and [32P]CTP from a pcDNA3-CETP construct that contained a fragment of human CETP cDNA spanning from 205 to 727 nucleotide of the coding sequence. An antisense riboprobe synthesized to the 3’ end of human actin was used to normalize RNA levels.

Reduction of CETP secretion with antisense oligonucleotides - CETP oligonucleotides A and B, corresponding to positions 291-311 and 359-379 of the human CETP mRNA coding sequence (6), respectively, were synthesized as follows: Oligonucleotide (A): sense - 5’-GAGCCAGCTACCCAGATATCA-3’; antisense - 5’-TGATATCTGGGTAGCTGGCTC-3’.

Oligonucleotide (B): sense - 5’-CACAACATCCAGATCAGCCAC-3’; antisense - 5’-GTGGCTGATCTGGATGTTGTG-3’.

The synthetic oligodeoxyribonucleotides, which were phosphorothioate modified and HPLC purified (Genosys), were dried and resuspended in tris-EDTA (10 mM tris, 1 mM EDTA, pH 7.4) and quantified by spectrophotometry. SW872 cells were transfected with oligonucleotides using lipofectamine according to the manufacturer’s protocol (Gibco BRL). Briefly, cells at 70 % confluence were transfected with Opti-MEM medium containing lipofectamine alone or mixed with sense or antisense oligonucleotide for 5 hr. Medium containing 10% FBS was then added and the cells were incubated for 24 hr. This media was then removed and replaced by DMEM/F12 media alone, incubated for 48 hr and used for CE transfer assay. Different concentrations of oligonucleotide (100 nM to 1 µM) were tested.

Effect of impaired CETP production on cellular lipid homeostasis - To assess the impact of reduced CETP secretion on free cholesterol, CE and TG biosynthesis, SW872 cells were transfected as described above then pretreated with DMEM/F12 media containing 5% LPDS for overnight. The cells were then incubated with DMEM/F12 media containing additional sense or antisense oligonucleotide (500 nM) and 14C-acetate (0.5 µCi/well) for 6 hr. This media was removed and replaced with fresh DMEM/F12 media containing 1% BSA. In some experiments, cells were treated with oligonucleotide and 14C-acetate (0.5 µCi/well) for 3 days in order to achieve near-isotopic equilibrium labeling of cellular CE pools in cells.
where CETP was suppressed long-term. After either treatment protocol, the cells were washed extensively with PBS, trypsinized, solubilized in 1 ml PBS and sonicated. Lipids were extracted according to the method described by Bligh and Dyer (31) and fractionated on thin layer chromatography using a mixture of hexane/diethyl ether/acetic acid (70:30:1). Radiolabeled CE, free cholesterol and TG, identified by co-migration with authentic lipid standards, were scraped from the plate and quantitated.

Cellular lipid efflux experiments in SW872 cells were performed by prelabeling control cells for 3 days in medium containing 10% fetal bovine serum and 3H-cholesterol (0.5 µCi/ml). Cells were washed 3 times with PBS and then transfected with Opti-MEM containing 500 nM sense or antisense oligonucleotide as described above. This medium was removed after 24 hr and fresh efflux medium containing 100 µg/ml of human HDL and additional sense or antisense oligonucleotide was added for an additional 24 hr. After removing this medium, cells were washed extensively with PBS, trypsinized and sonicated. Lipids in cells and the efflux media were extracted and separated as described above.

**Analytical Methods**

Cholesterol synthesis rates were determined after preincubation of cells in LPDS for 24hr. Washed cells received 300 µM 14C-acetate (1340 cpm/nmole) in DMEM/F12 media. After incubation, cellular lipids were extracted and free cholesterol was isolated by thin layer chromatography as described above. The synthetic rates reported are the average of 3 time points over the linear response range (d 6 hr). Neutral cholesteryl ester hydrolase activity in whole-cell lysates was determined from the hydrolysis of cholesteryl-(1-14C)oleate (New England Nuclear) incorporated into phosphatidylcholine / taurocholate vesicles (32). Hydrolysis was stopped by the addition of NaOH. Liberated, radiolabeled fatty acids were extracted and quantitated (33). Hydrolysis was linear for 50 - 300 µg cell lysate protein (t= 1 hr).

To quantify CE mass, treated cells were washed extensively with PBS and cellular lipids were extracted (31). Cholesteryl heptadecanoate (as internal standard) was added to each sample prior to extraction. Lipids were fractionated by thin layer chromatography (see above). The CE band was scraped into a reaction tubes and transesterified with BF3 as described by Sattler, et al. (34). The resultant fatty acid methyl esters were extracted, dried under N2, and resuspended in 20 µl hexanes. One-µl of this solution was separated and quantified by gas chromatography (35). CE mass was calculated from the mass of fatty
acid determined by this method plus the corresponding mass of the sterol ring.
RESULTS

Properties of SW872 Cells

CETP is expressed by a wide variety of tissues, including adipocytes (22). To examine the interrelationships of CETP expression and lipid metabolism, we selected the SW872 liposarcoma as a representative of this tissue type. SW872 cells, previously reported to secrete CETP (21), are deficient in lipid storage droplets when grown in serum containing medium (Figure 1A). The addition of oleate to the growth media, in the absence of agents required for cellular differentiation (i.e., hydrocortisone, insulin, etc. (36)), results in the rapid accumulation of triglyceride-containing droplets. Most all cells contain numerous small, lipid-filled inclusions 24 hr after oleate addition (Figure 1B); after 48 hr, lipid storage droplets fill the bulk of the cytoplasm (Figure 1C). Lipid accumulation is accompanied by a marked increase in cellular perilipin A (Figure 1D) which decorates the surface of lipid storage droplets in mature adipocytes (37). However, unlike lipid-laden adipocytes isolated from tissue, cholesterol biosynthesis in native SW872 cells is robust (2.5 nmoles acetate incorporated into cholesterol/mg protein/hr following 24 hr LPDS preincubation) compared to that observed in LPDS-treated HepG2 hepatocytes (5.2 nmoles/mg protein/hr). Overall, these observations indicate that SW872 is a fully mature, lipid-poor adipocytic cell line.

SW872 cells actively secrete CE transfer activity into the media. Compared to two other cell lines of human origin that have been reported to secrete CETP, CE transfer activity secreted by SW872 cells exceeds that produced by confluent cultures of HepG2 or Caco-2 cells (157.5 ± 12.7, 31.5 ± 12.0 and 92.7 ± 8.7 %kt/ml, respectively). The low CE transfer activity secreted by HepG2 cells is consistent with that previously reported, which may reflect loss of essential regulatory factors during culture (38). CE transfer activity secreted by SW872 cells is reduced 79.2% by anti-CETP antibodies (Figure 1E). This level of inhibition is identical to the maximum suppression that could be achieved by this antibody with isolated plasma CETP (Figure 1E), demonstrating that essentially all CE transfer activity in conditioned media is due to CETP. CETP secretion by confluent cultures of SW872 cells was nearly linear over 48 hr of culture (Figure 1F). Subsequent studies were restricted to this linear response window.

Perturbation of cellular lipid content alters CETP secretion
Dietary cholesterol has been shown to increase plasma CETP levels in different species (19,39,40). It has also been reported that dietary fatty acids can modulate CETP synthesis (41-43). To determine whether CETP biosynthesis and secretion in SW872 cells is modulated by variations in cellular lipid levels, cells were incubated with different compounds known to increase or decrease cellular lipid content.

Following a 24 hr pre-treatment with LPDS, cells were incubated with various sources of lipids for 24 hr. Subsequently, cells were incubated in media alone (48 hr) to collect media for measurement of CETP secretion without interference of the test agents on the CETP assay. Compared to cells incubated in media alone, incubation of cells with oleate (500 µM) stimulated CETP secretion by 3.8-fold (Figure 2A). Incubation of SW872 cells with a source of cholesterol also increased CETP secretion. Native (100 µg/ml) and acetylated human LDL (50 µg/ml) induced a 2- to 6-fold increase in CETP activity secreted into conditioned medium. These increases in secreted CETP activity were accompanied by similar changes in CETP protein (Figure 2A, inset, shown for oleate and LDL only). The effect of lipoproteins could be mimicked in large part by incubation with 25-hydroxycholesterol (100 µM), strongly suggesting that the influence of lipoproteins on CETP secretion is mediated through their modification of sterol metabolism. The same qualitative response to these agents was observed with Caco-2 cells (Figure 2B).

The effect of oleate on CETP secretion was concentration-dependent over a 50 to 500 µM range (data not shown). To investigate the response of CETP secretion to TG accumulation, SW872 cells were continuously incubated with oleate (200 µM) up to 4 days prior to the collection of 48 hr-conditioned media for CETP determination. Although CETP secretion by control cells increased slightly during the experiment, CETP secretion within the 48 hr collection window increased dramatically in oleate treated cells (Figure 2C). Cells exposed to oleate for 4 days secreted 7-fold more CETP during the 48 hr chase period than cells treated with the fatty acid for 2 days. The rate of CETP synthesis (%kt/hr) correlated well with the amount of cellular TG, suggesting that CETP biosynthesis is progressively upregulated in response to the accumulation of this lipid (Figure 2C, inset).

The above data demonstrate that CETP secretion is upregulated under conditions of cholesterol delivery where cholesterol biosynthesis is diminished. To determine whether CETP secretion is responsive to reductions in cell lipid content, cell were grown in medium containing 10% FBS until they reached 100% confluence, then switched to media that would stimulate cholesterol efflux from cells.
Following incubation of cells with media containing 5% LPDS, the cholesterol-binding compound β-cyclodextrin (500 µM) or a mixture of both of these agents for 24 hr, CETP activity secreted by cells was reduced by almost 60% (Figure 2D). CETP protein in the media mirrored the changes in CETP activity (inset).

To evaluate whether the observed changes in CETP expression by SW872 cells were due to alterations in mRNA levels, CETP mRNA levels were determined on cells immediately following the 24 hr treatment with the test compound. We observed that CETP mRNA levels were markedly altered by the various treatments, with the overall pattern of mRNA changes mirroring those noted for CETP secretion (Figure 3). These results suggest that altered CETP secretion is achieved by changes in CETP biosynthesis secondary to changes in CETP message levels.

**Reduction of CETP secretion by antisense oligonucleotide**

CETP synthesis is common among tissues involved in lipid storage and transport (4). Additionally, in species with circulating CETP (44) these tissues also synthesize a poorly-secreted truncated form of the transfer protein (10). Given this, and the close association between lipid metabolism and CETP synthesis/secretion noted above, we hypothesized that CETP may have a local role in cellular lipid metabolism. To investigate this possible novel role for CETP, we studied the influence of altered CETP synthesis on lipid metabolism.

Transfecting SW872 cells with antisense oligonucleotides targeting human CETP mRNA disrupted the biosynthesis of CETP. Cells were transfected with medium containing lipofectamine alone or lipofectamine plus sense or antisense oligonucleotide. CETP activity in conditioned medium (48 hr post-oligo treatment) was not significantly affected by transfection with lipofectamine or with 500 nM sense oligonucleotide (Figure 4). However, CETP secretion by antisense oligonucleotide A-transfected cells (oligo A) was reduced by 60% compared to sense oligonucleotide treatment. At the same concentration, antisense oligonucleotide B reduced CETP secretion by 40% (not shown). Unless specifically noted, subsequent studies used antisense oligonucleotide A.
Reduction of CETP biosynthesis modifies cellular cholesterol metabolism

In order to assess the effect of low CETP synthesis on cholesterol metabolism, transfected cells were pretreated with LPDS for 24 hr then labeled with 14C-acetate for 6 hr. In CETP-deficient cells, cholesterol biosynthesis was reduced by 20%, a small but statistically significant decline (Figure 5A). Interestingly, even though the cellular content of newly synthesized free cholesterol was lower, the incorporation of radiolabeled acetate into CE was increased by 30% in cells expressing lower CETP (Figure 5B). Acetate incorporation into total cholesterol (free cholesterol + CE) was also lower in CETP-deficient cells (p < 0.015), showing that the increased radiolabel in CE was not simply due to a redistribution of labeled cholesterol between these two pools. These changes in cholesterol metabolism were not reflected in TG synthesis (Figure 5C), showing that CETP suppression did not modify all lipid pathways.

Lower cholesterol synthesis combined with higher CE radioactivity suggests that the increased acetate incorporated into CE reflects the esterification of newly synthesized, radiolabeled fatty acids into an existing CE pool via the cholesterol ester esterase / cholesterol acyl transferase pathway (45). Alternatively, cholesterol could be more actively converted to CE in CETP-deficient cells. In either case, this short-term labeling study suggests abnormalities in CE metabolism when CETP levels are decreased.

To investigate this further, cells were labeled with 14C-acetate for 3 days during which time CETP biosynthesis was continuously suppressed by repetitive additions of antisense A oligonucleotide. Under these conditions, the accumulation of radiolabel in CE was markedly increased (3-fold) in CETP-suppressed cells (Figure 6A), strongly suggesting a link between CE metabolism and CETP expression. Similar results were observed in antisense oligonucleotide B treated cells (Figure 6B). These effects on CE were not due to differences in the radiolabel contained in the fatty acid precursor pool, as acetate incorporation into this lipid in sense- and antisense-treated cells was not statistically different (2.1 ± 0.2 vs. 1.8 ± 0.1 x 10^4 cpm/ mg protein, respectively). The increased CE pool, determined by radiolabeled incorporation, was confirmed by direct mass measurement. After 3 days of antisense A treatment, CETP-suppressed cells contained 2.5-fold more CE mass than cells that received the sense oligonucleotide (Table 1). This increase, which was observed in each of the three measurable CE species (Table 1), reflected a rise in CE from 6.3% to 15.5% of total cellular cholesterol (CE / total cholesterol).
To investigate the association of CE metabolism and CETP further, control cells were pre-incubated (3 days) with $^3$H-cholesterol to label cellular pools of free and esterified cholesterol. Subsequently, labeled cells were incubated for 24 hr with sense or antisense oligonucleotides. Fresh media containing the oligonucleotide and HDL (100 µg/ml) was then added and the efflux of cellular cholesterol pools to the HDL acceptor was determined after 24 hr. At time zero (before HDL addition), cells contained 13.8% of the incorporated cholesterol label in the CE pool. Treatment of cells with antisense oligonucleotides was without effect on the capacity of cells to efflux free cholesterol to HDL compared to sense control (2.26 ± 0.02 versus 2.56 ± 0.13 x 10^4 cpm/mg cell protein, respectively). In each instance, approximately 40% of the labeled free cholesterol was removed during the efflux phase of the experiment. In contrast, in CETP-suppressed cells the loss (hydrolysis) of radiolabeled CE induced by HDL was <50% of that in sense-treated cells (Figure 6C). In a separate, similar experiment, measurements of cellular CE mass by gas chromatography supported these findings. In sense-treated cells, 64% of CE was hydrolyzed during a 10 hr incubation with HDL, whereas in CETP-suppressed cells only 27% of the CE pool was degraded (Figure 6D). This was not due to a lower capacity of these cells to hydrolyze CE, since neutral cholesteryl ester hydrolase levels, measured with exogenous substrate, were not different (1.89 ± 0.21 versus 1.78 ± 0.23 nmoles CE hydrolyzed/mg cell protein/hr (sense versus antisense, respectively)). Thus, partial suppression of CETP synthesis is accompanied by a reduced capacity to mobilize cellular CE stores. Together, this finding, and the increased CE content of cells incubated for 3 days with antisense oligonucleotide shown above, strongly support the conclusion that CETP and cholesterol metabolism are interconnected in SW872 cells and suggests that CETP may play an important role in the normal trafficking of cellular cholesterol.
DISCUSSION

Most of our understanding of the mechanisms regulating CETP expression derives from in vivo studies of transgenic animals and correlation analyses of CETP levels with plasma lipid levels. One of the challenges to the study of CETP gene regulation in isolated systems has been the paucity of suitable cell models. Even though several cell lines derived from different tissues have been reported to synthesize and secrete CETP (46,47), the amount of CETP produced is often near detection limits and sometimes poorly responsive to regulatory stimuli (20,38). An exception to this generalization is the recent report that the SW872 adipocytic cell line secretes significant levels of CETP, which is upregulated by LDL or 25-OH cholesterol (21). Since adipose tissue is a CETP synthesis site common to all animals expressing this transfer protein in their plasma (4), we have investigated whether this cell line is a robust model in which the regulation of CETP biosynthesis can be studied.

A common feature of CETP biosynthesis identified through multiple approaches is its upregulation by cholesterol. High cholesterol diets increase plasma CETP levels in humans and hamsters, which are associated with an increase in CETP mRNA in liver, spleen, heart and adipose tissue (4,40,48). Similarly, plasma CETP concentrations are increased in certain hyperlipoproteinemic conditions such as chylomicronemia and dysbetalipoproteinemia (49). The increase in plasma CETP associated with these conditions may reflect enhanced delivery of lipoprotein-derived cholesterol to responsive tissues, such as adipose, where CETP gene expression is upregulated (22,40,49). In the present study we examined the responsiveness of CETP synthesis by cultured adipocytic cells to cholesterol. When incubated with native human LDL or acetylated LDL, CETP mRNA levels and secreted CETP activity and mass were significantly increased. Likewise, addition of non-lipoprotein associated 25-hydroxycholesterol to cells also increased CETP mRNA and CETP secretion. CETP secretion by Caco-2 cells was similarly sensitive to regulation by these agents. Together, these results indicate that irrespective of the mechanism of entry, cholesterol uptake by cells stimulates CETP biosynthesis.

The tight correlation between CETP biosynthesis and cellular sterol is further demonstrated by our observation that cells incubated under conditions that promote cholesterol efflux suppresses CETP secretion. This was observed with both lipoprotein-deficient plasma and the cholesterol-absorbing agent, β-cyclodextrin. The exact mechanism by which cholesterol regulates CETP gene expression is
still a matter of debate and is yet to be fully clarified. Data thus far suggest that the trans-activating factor SREBP-1 contributes to basal CETP expression on a chow diet (50), whereas dietary cholesterol regulation involves LXR and RXR interactions with a DR4 promoter element (51). The reproducible response of CETP biosynthesis to cholesterol in SW872 cells suggests that this cell line may be a suitable model to study the physiological regulation of CETP by sterols and to further the identification of transcription factors and response elements involved in this process.

Marked CETP mRNA and protein/activity upregulation was also observed with fatty acid addition to cells. This is consistent with a previous report that transcription of the CETP gene increases after challenging cells with sodium oleate (52). We further observed that CETP secretion was progressively increased as cells accumulated TG mass. These data illustrate that CETP biosynthesis is also increased under conditions where its other transfer substrate, TG, is increased. The specific mechanism(s) by which fatty acid may regulate CETP gene expression is not known yet. It is notable that CETP production in SW872 cells is strongly upregulated by sterol delivery and TG accumulation, whereas lipid-laden, tissue-derived adipocytes have a comparatively muted response to cholesterol and their CETP expression is negatively correlated with TG content (22). We suggest that the unique response of SW872 cells typifies mature adipocytes early in their progression to lipid-filled storage depots. This unique phenotype may be re-expressed as the lipid content of adipocytes wanes during the course of normal physiology.

The regulation of CETP expression and secretion by conditions that influence cellular levels of CETP substrates (TG and CE) suggests that CETP activity may be important in the transport of these lipids. This potential relationship was examined in short-term studies where CETP biosynthesis was partially suppressed by antisense oligonucleotide administration. Acute reduction in CETP synthesis by 50-60% resulted in a small, yet significant reduction in cholesterol synthesis, but more than a 30% increase in acetate incorporation into the CE pool. TG synthesis was unaffected. Increased CE radioactivity in the face of decreased cholesterol synthesis indicates that the CE pool is labeled primarily through the incorporation of radiolabeled fatty acids during the deacylation/reacylation cycle of this sterol pool. These data suggest that the CE pool in CETP-compromised cells is increased. This was subsequently demonstrated in studies where CETP synthesis was suppressed for an extended time (3 days). Here, the CE pool, measured by either isotope incorporation or direct mass determination, was
increased ~3-fold in CETP-suppressed cells. Similar results were obtained with two different antisense oligonucleotides, supporting the conclusion that suppression of CETP synthesis compromises cellular metabolism of CE. Increased CE could arise from increased esterification of free cholesterol or ineffective mobilization of CE for efflux. We tested the latter possibility by pre-labeling normal cells with 3H-cholesterol, treating cells with sense or antisense oligonucleotide, then measuring the loss of radioactivity from the CE pool when HDL was added to the medium. Cells with reduced CETP expression demonstrated a significant reduction in the capacity to mobilize CE compared to control. CE mass measurements showed a similar deficiency in CE hydrolysis. This suggests that the accumulation of CE in antisense treated cells is, at least in part, due to a defect in CE hydrolysis. Failure to hydrolyze CE, however, is not due to altered neutral CE hydrolase activity since its activity was unchanged by oligonucleotide treatment.

Overall, these studies demonstrate that CETP and cellular lipid metabolism are integrally linked. While confirming and extending current understanding of how CETP synthesis is regulated by factors influencing cellular lipid levels, we demonstrate for the first time that CETP activity is important for normal sterol trafficking in SW872 cells. The mechanisms by which CETP modulates cellular cholesterol metabolism are yet to be determined and are presently under investigation. The primary phenotype of CETP-deficient cells is an expanded CE pool. We speculate that the accumulation of CE in CETP-compromised cells occurs because this lipid is poorly translocated from its site of synthesis (microsomes) to storage droplets were it can be degraded by the neutral CE hydrolase. Since microsomal triglyceride transfer protein has low specificity for CE (53), it is interesting to speculate that CETP may have such an intracellular role in CE transport. CETP exists in two forms, a full-length form that is normally secreted, and a truncated form derived from alternative splicing (10). The short form of CETP is poorly secreted but retains the CE/TG binding sites and lipid-surface interaction sites required for lipid transfer activity (11). In adipocytes, intracellular CETP co-isolates with several purified membrane fractions including microsomes (48). And finally, CETP has broad specificity for membrane surfaces and can transfer CE from biological membranes including the endoplasmic reticulum (54). A localized function for CETP, especially the non-secreted shorter form, in facilitating cellular sterol metabolism and storage would help explain why the CETP gene and CETP-like mRNA are found in the tissues of animals that do not have circulating CETP (4) and why CETP synthesis is strongly influenced by the lipid status of cells. On-going
studies where CETP activity is more completely suppressed for an extended time should provide an opportunity to more rigorously examine this novel function for CETP.
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ABBREVIATIONS - CETP, cholesteryl ester transfer protein; LDL, low density lipoprotein; HDL, high density lipoprotein; CE, cholesteryl ester; TG, triglyceride; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; LPDS, lipoprotein-deficient serum.
Table 1

**Modification of cellular cholesteryl ester mass in cells treated with CETP antisense oligonucleotide**

SW872 cells were incubated in serum-free media containing the indicated oligonucleotide (500 nM) for three days. Cells were washed extensively and lipids were extracted. Cholesteryl esters were isolated by thin layer chromatography, transmethylated, and the resulting fatty acid methyl esters were quantitated by gas chromatography. See Methods for details. Values are mean ± S.D. The increases in total CE and individual CE species in antisense-treated cells were significant at the P< 0.01 level.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C18:1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense (n=6)</td>
<td>0.53 ± 0.11</td>
<td>0.28 ± 0.20</td>
<td>1.11 ± 0.45</td>
<td>1.93 ± 0.51</td>
</tr>
<tr>
<td>Antisense (n=5)</td>
<td>1.42 ± 0.57</td>
<td>0.90 ± 0.25</td>
<td>2.40 ± 0.74</td>
<td>4.72 ± 0.78</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1: Triglyceride accumulation and CETP secretion in SW872 cells - SW872 cells grown to 100% confluence in DMEM/F12 containing 10% FBS were incubated with DMEM/F12 ± 400 µM oleate for up to 48 hr. SW872 cells incubated without oleate (Panel A) or with oleate for 24 hr (Panel B) or 48 hr (Panel C; 2.5x greater magnification compared to Panels A and B) were stained with Oil Red O and hematoxylin/eosin as described (55). Panel D - Perilipin A immunoblot. Proteins from cells treated (48 hr) as described above were separated on 7.5% gels, transferred to PVDF, and reacted with guinea pig anti-perilipin antisera (see Methods). Y1 cells (positive control) are of mouse adrenal origin; HepG2 cells are negative control. Panel E - Inhibition of CETP activity in conditioned medium collected from SW872 cells or CETP purified from human plasma by excess TP2 monoclonal antibody (10 µg). Similar suppression was observed with 1 µg TP2. Panel E - Time course of CETP secretion by SW872 cells. The values are mean ± SD of duplicates. For details see the Methods.

Figure 2: Cellular lipid status modulates CETP secretion - The effect of lipid delivery to cells on CETP secretion by SW872 and Caco-2 cells is shown in Panel A and Panel B, respectively. Confluent cells pretreated with medium containing 5% LPDS overnight were treated with serum-free medium containing oleic acid (500 µM), LDL (100 µg/ml), acetylated-LDL (100 µg/ml) or 25-hydroxycholesterol (100 µM) for 24 hr. This media was removed and replaced by DMEM/F12 alone and incubated for 48 hr. Conditioned media was assayed for CETP activity. Panel A Inset - Western blot of immunoprecipitated CETP present in the conditioned media after the indicated treatment. Panel C - Cells, preincubated in 5% LPDS, were treated without (open squares) or with oleate (200 µM, closed squares) for the times indicated. This media was removed and DMEM/F12 media added for 48 hr. This conditioned media was assayed for CETP activity. Inset - Rate of CETP synthesis (%kt/hr) versus the triglyceride content of cells, determined by enzymatic assay. Panel D - CETP activity secreted by SW872 cells after treatment for 24 hr with medium containing 5% LPDS, β-cyclodextrin (500 µM) or a mixture of both. Cells were pretreated with serum-containing media overnight prior to the indicated treatment. Inset - Western blot of CETP in the conditioned media after treatment as indicated. The values are mean ± SD of duplicates, and are representative of at least 3 experiments. Abbreviations: cydex., cyclodextrin; Ac-LDL, acetyl LDL; 25-OH chol., 25-hydroxycholesterol.
Figure 3: Cellular lipid status regulates CETP mRNA levels in SW872 cells - SW872 cells at 100% confluence were pretreated with medium containing 5% LPDS for 24 hr, then subjected to the indicated treatment for another 24 hr. Total RNA was prepared after these treatments, 20 µg aliquots of RNA were separated on 8% SDS-polyacrylamide-urea gels, transferred onto a membrane, and analyzed by RNAse protection assay as described in the Methods. The bottom panel shows the densitometry values obtained for each band compared to actin. Abbreviations: Ac-LDL, acetyl LDL; 25 OH-chol, 25-hydroxycholesterol.

Figure 4: Reduction of CETP secretion levels in SW872 cells by antisense oligonucleotides - SW872 cells at 70% confluence were transfected with Opti-MEM alone, lipofectamine, or lipofectamine mixed with 500 nM sense or antisense oligonucleotide (A) made against human CETP mRNA. These cells were incubated with fresh DMEM/F12 for 48 hr. Collected medium was concentrated and 100 µl aliquot was used to measure CETP activity. The values are the mean ± SD of duplicates, and are representative of at least 3 experiments. Abbreviation: oligo (A); oligonucleotide (A).

Figure 5: Reduced CETP synthesis modifies cellular lipid metabolism in SW872 cells - SW872 cells, untreated (none) or transfected with lipofectamine containing 500 nM sense or antisense oligonucleotide (A) were incubated overnight in DMEM/F12/5% LPDS. Subsequently, washed cells received the same oligonucleotide-containing media containing 14C-acetate (0.5 µCi/well) for 6 hr. This medium was removed and replaced with DMEM/F12 + 5% LPDS overnight. Label incorporated into free cholesterol (Panel A), cholesteryl ester (Panel B) and triglyceride (Panel C) were determined as described in the Methods. The values are the mean ± SD of triplicates.

Figure 6: Effect of low CETP on cholesteryl ester content and efflux - SW872 cells at 70% confluence were treated with lipofectamine containing 500 nM oligonucleotides as indicated in the Methods, then incubated with 14C-acetate (0.5 µCi/well) for 3 days. During this incubation, oligonucleotides were added daily to the cells. The labeled CE content of cells after treatment with antisense oligonucleotide A (Panel A) or antisense oligonucleotide B (Panel B) is shown. In Panel C, control cells were incubated with 3H-cholesterol for 3 days in medium containing 10% fetal bovine serum. Labeled cells were transfected
with sense or antisense oligonucleotides (500 nM) as described in the Methods. After overnight incubation, cells received serum-free efflux medium containing 100 µg/ml HDL plus additional sense or antisense oligonucleotide. After 24 hr, cellular lipids were extracted and 3H-cholesteryl ester was determined. Hydrolysis was calculated from the decline in CE radioactivity at 24 hr compared to that at t=0 (9.5 x 10^3 cpm/mg protein). Values are the mean ± SD of triplicates. In Panel D, cells were incubated as indicated for Panel C except that radiolabeled cholesterol was not added. Following 24 hr treatment with the indicated oligonucleotide (t=0) and after incubation (10 hr) with HDL (100 µg/ml), cells were harvested, lipids extracted, and cholesteryl esters were isolated by thin layer chromatography. Cholesteryl esters were transmethylated and quantitated by gas chromatography. Results are the mean ± SD of n= 5 (sense) or 6 (antisense) replicates. The cholesteryl ester content of t=0 cells (post 24 hr treatment with oligonucleotide) was not significantly different between sense and antisense treatment.
Figure 4

The graph shows the CETP activity in different treatments. The treatments include Opti-MEM, Lipofectamine, Oligo (A) Sense, and Oligo (A) Antisense. The CETP activity is measured as a percentage of total activity (%kt). The graph indicates a significant difference in CETP activity across the treatments.

Senac Antisense
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