Intracellular lipid droplets (LDs) are composed of a neutral lipid core of triglyceride (TG) and cholesterol esters (CEs) that is surrounded by a monolayer of phospholipids and embedded proteins. Fatty acids and cholesterol are stored or released from LDs in response to hormonal stimuli that regulate phosphorylation of perilipins and lipases [reviewed in (1)] or by a specialized type of autophagy termed lipophagy (2). The provision of LD-derived fatty acids and cholesterol is critical for membrane biogenesis, hormone synthesis, energy production, and lipoprotein secretion. The surface monolayer of phospholipid and cholesterol is an important structural component of LDs that stabilizes and controls their capacity to store and mobilize lipids (3–5). Phosphatidylcholine (PC) is the most quantitatively significant component of LDs and, depending on the mammalian cell type, is produced by three different biosynthetic pathways; the CDP-choline pathway, phosphatidylethanolamine (PE) methylation, and Lands pathway lysophosphatidylcholine acyltransferases (LPCATs). All three pathways are important for providing the PC required for LD biogenesis and expansion (6–9).

The CDP-choline pathway synthesizes the bulk of PC for cellular membranes and secretion, and supplies PC for LD expansion and stabilization in mammalian cells [reviewed in (10)]. The terminal enzymes in the CDP-choline pathway (Fig. 1A), choline phosphotransferase (CPT) and choline/ethanolamine phosphotransferase (CEPT), produce PC from CDP-choline and diacylglycerol in the ER and Golgi apparatus (11). However, the rate-limiting step in the pathway is catalyzed by CTP:phosphocholine cytidylyltransferase (CCT)α and CCTβ isoforms, which are soluble enzymes that undergo activation upon translocation to membranes in response to lipid activators, such as fatty acids (i.e., oleate) (12, 13). CCTβ is a tissue-specific isoform that translocates from the cytoplasm to the ER where it produces CDP-choline in the vicinity of ER-localized CPT (14, 15). The more abundant ubiquitously expressed CCTα

**Abstract**

Intracellular lipid droplets (LDs) supply fatty acids for energy, membrane biogenesis, and lipoprotein secretion. The surface monolayer of LDs is composed of phospholipids, primarily phosphatidylcholine (PC), that stabilize the neutral lipid core of triglyceride (TG). To determine the relationship between PC synthesis and TG storage and secretion in chylomicrons, we used a model of intestinal-derived human epithelial colorectal adenocarcinoma (Caco2) cells with knockout of PCYT1A, which encodes the rate-limiting enzyme CTP:phosphocholine cytidylyltransferase (CCT)α in the CDP-choline pathway, that were treated with the fatty acid oleate. CRISPR/Cas9 knockout of CCTα in Caco2 cells (Caco2-KO cells) reduced PC synthesis by 50%. Compared with Caco2 cells, Caco2-KO cells exposed to oleate had fewer and larger LDs and greater TG accumulation as a result. The addition of exogenous lysophosphatidylcholine to Caco2-KO cells reversed the LD morphology defect. Caco2-KO cells, differentiated into epithelial monolayers, accumulated intracellular TG and had deficient TG and chylomicron-associated apoB48 secretion; apoB100 secretion was cumulated intracellular TG and had deficient TG and chyomicron secretion. The surface monolayer of LDs is composed of phospholipid and secretion, and supplies PC for LD biogenesis and expansion (6–9).

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is a nucleoplasmic enzyme that is activated upon translocation to the nuclear envelope (16-18). During differentiation of 3T3-L1 preadipocytes, CCTα mRNA and protein expression is increased, and CCTα translocates to the nuclear envelope to increase PC synthesis that is required for LDL biogenesis (9). Insect cells offer a contrasting model of CCTα regulation. In olate-stimulated Drosophila S2 cells, the CCTα homolog is exported from the nucleus to the surface of LDs where increased CDP-choline synthesis drives PC production for biogenesis and expansion of the LD monolayer (8).

In the liver and intestine, the neutral lipids stored in LDs are hydrolyzed, reesterified, and incorporated into lipoproteins (19, 20). In hepatocytes, disruption of PC synthesis by nutritional approaches or knockout of enzymes in the CDP-choline or PE methylation pathways increases TG storage in LDs and inhibits the secretion of VLDL (21, 22). TG homeostasis in intestinal epithelial cells is also regulated by PC. Disruption of the Lands pathway for PC acyl-chain remodeling by Lpexit38 knockout in the intestine reduced the levels of arachidonyl-PC that is critical for lipid absorption (23, 24). However, the role of the CDP-choline pathway in enterocyte TG homeostasis has not been addressed. The pathway appears to be involved in TG secretion and storage because rats fed a choline-deficient diet displayed accumulation of intestinal neutral lipids and reduced circulating apoB48-containing lipoproteins (25). To establish the contribution of the CDP-choline pathway to the balance between TG storage in LDs and its secretion in lipoproteins by intestinal cells, we used CRISPR/Cas9 to delete PCYT1A in Caco2 cells. Here, we report that Caco2 CCTα-knockout (Caco2-KO) cells had decreased LD number, but increased LD size and TG mass, which was partially reversed by supplementation with lysophosphatidylcholine (lyso-PC) to stimulate PC synthesis by the Lands pathway. When differentiated into polarized monolayers, Caco2-KO cells also had larger LDs as well as decreased basolateral secretion of apoB48-containing chylomicrons and TG. Interestingly, the secretion of apoB100 was unaffected, indicating a specific requirement for the CDP-choline pathway in secretion of TG-rich chylomicrons.

MATERIALS AND METHODS

Cell culture

Wild-type and Caco2-KO cells were cultured in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (medium A) at 37°C in a 5% CO2 atmosphere. Caco2 cells were differentiated into polarized epithelial monolayers by culturing on polypropylene membrane inserts of 35 mm Transwell plates (Corning Costar, Cambridge, MA) for 21 days in medium A. Oleate/BSA complexes (6:1, mol/mol) were prepared as described (26). Lyso-PC stock solutions (25 mM) were prepared in ethanol.

The integrity of epithelial monolayers was determined by measuring transepithelial resistance (TER) in an Ussing chamber (aperture size of 0.12 cm2). Each hemichamber contained 1.2 ml Krebs solution [117 mM NaCl, 4.6 mM KCl, 20 mM NaHCO3, 6 mM glucose, 1 mM MgCl2, 1.5 mM CaCl2, and 10 mM HEPES (pH 7.4)]. Ag/AgCl electrodes were connected to the apical and basolateral side of the membrane inserts with agar bridges (3 M KCl-2% agar) to measure transepithelial potential difference. TER was determined by the change in the transepithelial potential difference generated by a brief 10 μA pulse controlled by high-impedance automatic dual voltage clamps. Resistance data were collected and analyzed using a PowerLab 28T and LabChart software (ADInstruments, Colorado Springs, CO).

CRISPR/Cas9 knockout of CCTα

PCYT1A was disrupted in Caco2 cells by CRISPR/Cas9 gene editing (27) using a guide RNA designed (http://crispr.mit.edu/) to target exon 3 (G-GCCCGGGACCCCAAC-3). The guide RNA was cloned in px459 and transfected into Caco2 cells followed by selection with puromycin for 7 days. Clonal cell lines were isolated by limiting dilution and screened for CCTα expression by SDS-PAGE and immunoblotting.

Isolation of lipoproteins by ultracentrifugation

Differentiated Caco2 cells were incubated with medium A containing oleate/BSA in the apical chamber. The basolateral medium A (0.8 ml) was collected into polycarbonate tubes (Beckman Instruments, Inc.), overlayed with a 1.006 g/ml solution [0.15 M NaCl and 0.3 mM EDTA (pH 7.4)], and subjected to centrifugation at 480,000 g for 2.5 h in an Optima™ TLX tabletop ultracentrifuge using a TLA-120.2 fixed-angle rotor (Beckman Instruments, Inc.), overlayed with a 1.006 g/ml NaBr solution and centrifuged for 4 h at 480,000 g. The apoB100 and apoB48 in the lipoprotein fractions were resolved by SDS-5%PAGE and immunoblotted for human apoB100 and apoB48 (see below).

Immunoblotting

Cell lysates or lipoprotein fractions were adjusted in 2x SDS buffer [12.5% SDS, 30 mM Tris-HCl, 12.5% glycerol, and 0.01% bromophenol blue (pH 6.8)], heated at 95°C for 5 min, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were incubated in TBS [20 mM Tris-HCl and 500 mM NaCl (pH 7.4)]; Odyssey blocking solution (5:1, v/v) for 1 h. Membranes were then probed with antibodies against CCTα (28), CCTβ2 (rabbit polyclonal; provided by S. Jackowski, St. Jude Children’s Research Hospital, Memphis, TN), β-actin (mouse monoclonal AC15; Sigma-Aldrich), or human apoB (mouse monoclonal 1D1; provided by Roger McLeod, Dalhousie University, Halifax, NS, Canada). Proteins were visualized with goat anti-mouse or goat anti-rabbit IRDye-800- or -680-conjugated secondary antibodies (LI-COR Biosciences) using an Odyssey imaging system and application software (v3.0; LI-COR Biosciences).

Analysis of PC synthesis using [3H]choline

PC synthesis was measured by incubating cells in choline-free medium A containing [3H]choline (2 μCi/ml) as previously described (29). After labeling, cells were rinsed once with cold PBS, harvested in methanol:water (5:4, v/v), and [3H]PC was extracted in chloroform. Water-soluble metabolites of [3H]choline were quantified after separation by TLC in ethanol:water:ammonia (48:95:6, v/v). Radioactivity in PC and choline metabolites was measured by liquid scintillation counting and normalized to total cellular protein.

Choline transport assay

Cells were rinsed twice in 0.5 ml of Krebs-Ringer buffer [130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl2, 1.5 mM MgCl2, 1.2 mM

PC synthesis and chylomicron secretion 1941
K$_2$HPO$_4$, 10 mM D-glucose, and 10 mM HEPES (pH 7.4) and incubated in 0.5 ml of the same buffer for 30 min at 37°C. Cells then received 0.5 ml of Krebs-Ringer buffer containing 1-25 μM $[\text{H}]$choline for 10 min at 37°C. Cells were placed on ice, washed three times with ice-cold Krebs-Ringer buffer, and solubilized in 250 μl of 0.1% SDS/0.2 N NaOH for 10 min at room temperature. Radioactivity in 50 μl of sample was measured by liquid scintillation counting and expressed relative to total cell protein.

**Analysis of TG mass and metabolism**

TG synthesis by undifferentiated Caco2 cells was measured by incubation with 2 μCi $[\text{H}]$glycerol and 200 μM oleate/BSA for up to 6 h in medium A. Cells were rinsed twice with cold NaCl (150 mM), Tris-HCl (pH 7.4) (50 mM), and BSA (2 mg/ml), and once with cold NaCl (150 mM) and Tris-HCl (pH 7.4) (50 mM). The $[\text{H}]$glycerol-labeled lipids were extracted directly from the dish with hexane/isopropanol (3:2, v/v) and separated by TLC in hexane:diethyl ether:acetic acid (90:30:1, v/v). Radioactivity in TG was measured by liquid scintillation counting and normalized to total cellular protein.

TG mass was quantified in cells incubated in the presence or absence of oleate/BSA using a TG quantification kit (BioVision Inc., San Francisco, CA). The method involves the colorimetric detection of glycerol released enzymatically from cellular TG.

TG and phospholipid synthesis and secretion in differentiated Caco2 cells was determined by incubation of the apical chamber with medium A containing 200 or 400 μM $[\text{H}]$oleate/BSA. After 12 h, medium was removed and cells were rinsed in cold PBS. Cells and medium were extracted with hexane/isopropanol and $[\text{H}]$oleate incorporation into TG and CE was determined as described above.

Cells were incubated with apical medium A containing $[\text{H}]$glycerol (2 μCi/ml) and 400 μM of oleate/BSA for 12 h. Basolateral medium was collected, cells were rinsed with medium A, and incorporation of radioactive glycerol into intracellular and secreted TG and phospholipids was determined after extraction with hexane/isopropanol (0 time). The turnover of $[\text{H}]$glycerol-labeled lipids was determined by incubation in medium A and isolation of $[\text{H}]$glycerol-labeled TG and phospholipids from the medium and cells at 3 and 6 h. TG was separated by TLC in hexane:diethyl ether:acetic acid (90:30:1, v/v) and phospholipids were separated by TLC in chloroform:methanol:acetic acidwater (60:10:4:1, v/v). Radioactivity was measured by liquid scintillation counting and normalized to total cellular protein.

**Immunofluorescence microscopy**

Cells cultured on glass coverslips were fixed in 4% (v/v) paraformaldehyde and permeabilized for 10 min in 0.05% (w/v) Triton X-100 at 4°C. In the case of polarized cells, Transwell membrane inserts were fixed in 4% (w/v) paraformaldehyde and permeabilized with 0.2% (w/v) Triton X-100 at room temperature for 30 min. Cells on glass slides or Transwell filters were incubated with a CCTX rabbit polyclonal antibody (28) and lamin A/C, LMNA/C monoclonal (4C11, Cell Signaling) in PBS containing 1% (w/v) BSA. This was followed by secondary Alexa-Fluor-conjugated goat anti-rabbit and anti-mouse secondary antibodies (Thermo Fisher Scientific). Coverslips or Transwell membranes were mounted on glass slides in Mowiol 4,88, and confocal imaging was performed using a Zeiss LSM510/Axiscope 200M microscope with a Plan-Apochromat 63x/1.40 numerical aperture oil immersion objective. LD size distribution was quantified using ImageJ software, version 1.47 (National Institutes of Health). Images were converted to 8-bit, the threshold was adjusted, and the “analyze particle” command was used to exclude cells at the edges. The percent distribution of LDs within a binned area group was determined.

**LCMS/MS analysis of PC molecular species**

A Dionex Ultimate 3000 UHPLC system coupled to a Thermo Scientific LTQ-Orbitrap Velos Pro mass spectrometer equipped with an electrospray ionization source was used. Lipids were separated on a Waters BEH C8 UPLC column (1.7 μm, 2.1 × 50 mm) using 0.01% formic acid in water and acetonitrile-isopropanol (1:1)-0.01% formic acid as the mobile phases for binary-gradient elution. The column flow rate was 0.4 ml/min and the column temperature was 60°C. For metabolite detection and relative quantitation, the instrument was run in the survey scan mode with FTMS detection at a mass resolution of 60,000 FWHM at m/z 400. The mass scan range was m/z 150–1,800. For LC-MS/MS, collision-induced dissociation was applied at normalized collision energies of 28–35% in a data-dependent analysis. Metabolite assignments were carried out by searching against the lipid and metabolome databases with the measured m/z values within an allowable mass error of ±3 ppm. LC-MS/MS using collision-induced dissociation in the ion trap was performed to provide confirmation of the assigned lipid identities. The survey scan was performed in a mass range of m/z 200–1,800 and the top six most intensive ions on each survey scan were selected for subsequent fragmentation at normalized collision energies of 28–35%. MS/MS spectra were manually interpreted to confirm the lipid classes and identities by the observed fragment ions, especially those class-specific characteristic ions, e.g., 184.073 [phosphocholine (pCholine)] and 104.10 (choline) for PC species. Peak areas for PC species were normalized to total cell protein.

**RESULTS**

**Increased TG storage in Caco2-CCTα knockout cells**

Caco2 cells are a human epithelial colorectal carcinoma that differentiates into polarized monolayers when cultured on permeable filter supports. Differentiated Caco2 cells have features of absorptive brush border intestinal epithelial cells, actively importing fatty acids at the apical surface for TG synthesis and secreting TG-rich apoB48-containing chylomicrons from the basolateral surface (30, 31). To assess whether PC synthesized by the CDP-choline pathway (Fig. 1A) was involved in TG storage and lipoprotein secretion, CRISPR/Cas9 gene editing was used to knock out the PCY1A gene that encodes CCTX in Caco2 cells. We isolated a Caco2-KO cell line that was devoid of CCTX protein and displayed no compensatory change in expression of the CTTβ2 isoform (32) (Fig. 1B). Immunofluorescence microscopy of undifferentiated Caco2 cells revealed that CCTX was expressed exclusively in the nucleus where it colocalized with LMNA/C (Fig. 1C). Exposure of the cells to 400 μM oleate/BSA for 12 h resulted in the accumulation of BODIPY-493/503-positive LDs, but did not affect nuclear localization of CCTX. Caco2-KO cells were devoid of nuclear CCTX and accumulated cytoplasmic LDs when exposed to oleate (Fig. 1D). However, compared with oleate-treated control cells (Fig. 1C), the knockout cells appeared to have fewer and larger LDs.

To assess the effect of CCTX knockout on PC synthesis, cells were pulse-labeled for up to 8 h in medium containing
and turnover in Caco2-KO cells can be explained by a 30–40% reduction in the proliferation of these cells (Fig. 2D). Thus, synthesis and degradation of PC are not linked in Caco2-KO cells, resulting in reduced cell proliferation due to PC deficiency.

LC-MS/MS analysis was performed to determine whether reduced PC synthesis and proliferation of Caco2-KO cells affected the mass of individual PC molecular species. The mass of major PC molecular species in undifferentiated (Fig. 3A) or differentiated (Fig. 3B) Caco2-KO cells was similar to controls. However, there was a notable shift in the distribution of PC to long-chain unsaturated species (i.e., 36:3, 38:4, and 38:5) when Caco2 and Caco2-KO cells were differentiated. The mass of PE molecular species was also unaffected by CCTα knockout in undifferentiated (Fig. 3C) or differentiated (Fig. 3D) Caco2 cells. Differentiation had a minimal effect on PE species, with a slight increase in the 38:4 species.

BODIPY-493/503-stained LDs were noticeably fewer and larger in Caco2-KO cells cultured in the presence of 400 μM oleate (Fig. 4A). Quantification of LD cross-sectional area in Caco2-KO cells confirmed a significant reduction in small LDs (<0.2 μm²) and an increase in LDs >1 μm² (Fig. 4B). Caco2-KO cells also had a 60% reduction in the total number of LDs per cell (Fig. 4C). The shift toward large

[^3H]choline, and isotope incorporation into PC and metabolites of the CDP-choline pathway were measured (Fig. 2A). The [^3H]choline incorporation into PC in Caco2-KO cells was lower than control values throughout the labeling period, reaching a significant 50% reduction at 6 h. Unexpectedly, isotope incorporation into pCholine, the substrate of CCTα, was not significantly affected in Caco2-KO cells. However, there was a variable reduction in the PC degradation product, [^3H]glycerophosphocholine, during the 8 h labeling period in Caco2-KO cells. Overall, Caco2-KO cells had a 40% reduction in [^3H]choline incorporation into all metabolites at 8 h, indicative of reduced flux through the CDP-choline pathway. The reduced incorporation of [^3H]choline into PC metabolites was not due to defective choline uptake (Fig. 2B). Maximal choline transport and the $K_t$ for control (138 ± 36 pmol/min/mg and 16 ± 8 μM) and Caco2-KO cells (146 ± 36 pmol/min/mg and 17 ± 8 μM) were similar. Despite a significant reduction in PC synthesis in Caco2-KO cells, the degradation of [^3H]choline-labeled PC was similar to controls during a 72 h chase period: the half-life for [^3H]PC in Caco2 cells was 46.1 ± 3.6 compared with 41.0 ± 5.4 in Caco2-KO cells (Fig. 2C). The apparent disconnect between PC synthesis and turnover in Caco2-KO cells can be explained by a 30–40% reduction in the proliferation of these cells (Fig. 2D). Thus, synthesis and degradation of PC are not linked in Caco2-KO cells, resulting in reduced cell proliferation due to PC deficiency.

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LDs in knockout cells treated with oleate was accompanied by a >3-fold increase in TG mass compared with control cells (Fig. 4D). The increase in TG mass in Caco2-KO cells appeared to be due to reduced mobilization because synthesis (measured by [3H]glycerol incorporation into TG) was similar to control cells (Fig. 4E). The synthesis of CE (measured by [3H]oleate incorporation) was also unaffected by CCTα knockout (Fig. 4F).

To verify that the abnormal LD phenotype in Caco2-KO cells (Fig. 4) was caused by CCTα knockout, we assessed whether it could be corrected by transient expression of mCherry-CCTα (supplemental Fig. S1). As expected, untreated or mock-transfected Caco2-KO cells had larger and fewer LDs. Expression of mCherry-CCTα in Caco2-KO cells reverted the LD distribution and number back to wild-type Caco2 levels (supplemental Fig. S1), indicating that the LD phenotype is due to loss of CCTα expression.

Esterification of lyso-PC by the Lands pathway enzymes LPCAT1 or -2 is involved in PC synthesis and acyl-chain remodeling on the surface of LDs (7, 33). Proteomic analysis also detected LPCAT2 on LDs from Caco2 cells (34). To determine whether PC synthesized by the Land pathway could substitute for the CDP-choline pathway, oleate-treated undifferentiated Caco2-KO cells were supplemented with 18:0-lyso-PC or 18:1-lyso-PC. Caco2-KO cells supplemented with 18:0-lyso-PC displayed a partial shift in size distribution of LDs toward that of control Caco2 cells (Fig. 5A, B). Supplementation with 18:1-lyso-PC was more effective in this regard, converting the LD size distribution in Caco2-KO cells to that of Caco2 controls. Similarly, 18:1-lyso-PC supplementation increased the number of LDs in Caco2-KO cells to a level that was similar to untreated controls (Fig. 5C). These data indicate that defects in LD size and number due to CCTα deficiency can be corrected by a LPCAT-scavenging pathway provided with sufficient exogenous lyso-PC.

**LD morphology in differentiated Caco2 cells**

When differentiated into epithelial monolayers on membrane supports, Caco2 cells take up and esterify fatty acids into TG for secretion in chylomicrons and VLDL-like particles from the basolateral surface (35). After differentiation of cells on Transwell inserts for 21 days, TER measurements showed that Caco2 (316 Ω·cm²) and Caco2-KO (323 Ω·cm²) formed tight epithelial monolayers. Untreated differentiated Caco2 and knockout cells contained more TG compared with undifferentiated counterparts (compare with Fig. 4D) and oleate treatment of differentiated cells for 12 h increased TG mass by 2- to 2.5-fold (Fig. 6A). Visual inspection of BODIPY-stained differentiated cells
revealed that untreated Caco2-KO cells contained fewer and larger LDs compared with untreated controls, and that exposure to 400 μM of oleate/BSA had minimal effect on LD distribution (Fig. 6B). It also appeared that incubation of Caco2-KO cells with oleate and 18:1-lyso-PC partially reverted the LD distribution to that of control cells. Quantitation of LD area confirmed a significant increase in the proportion of large LDs (>16 μm²) relative to small LDs (<2 μm²) in Caco2-KO cells (Fig. 6C). Note that the bins used to quantify LD size distribution in differentiated and undifferentiated Caco2 cells were different due to the LD size variation between the two groups (compare Fig. 4B and Fig. 6C). LD size in differentiated cells was not affected by incubation with oleate. However, incubation of Caco2-KO cells with oleate and 18:1-lyso-PC shifted the size distribution to that of control cells (Fig. 6C). The shift to large LDs in Caco2-KO cells was accompanied by a significant decrease in LDs per cell that was unaffected by exposure to exogenous oleate, but reverted to control levels when 18:1-lyso-PC was present (Fig. 6D). Thus, a deficiency in the CDP-choline pathway in differentiated Caco2 cells also caused a shift to fewer and larger LDs, which was corrected by incubation with exogenous 18:1-lyso-PC.

Reduced secretion of chylomicrons and TG by Caco2-KO cells

We next determined whether CCTα knockout affected lipid and lipoprotein secretion by differentiated Caco2 cells. First, the effect of CCTα knockout on TG metabolism in differentiated cells was determined by addition of [³H]oleate/BSA.
oleate/BSA to the apical medium and analysis of incorporation into basally secreted and intracellular TG and CE. Relative to controls, incorporation of 200 and 400 μM [3H] oleate into intracellular TG in Caco2-KO cells was significantly increased 1.8- and 2.9-fold, respectively (Fig. 7A). Cellular accumulation of [3H]TG was accompanied by a 2.4- and 2.0-fold reduction in TG secretion by Caco2-KO cells (Fig. 7A). Oleate incorporation into intracellular CE was also increased in Caco2-KO cells, but secretion was not affected (Fig. 7B). Thus, CCTα knockout leads to intracellular accumulation and impaired secretion of TG.

The metabolism of TG in Caco2-KO cells was also monitored by labeling with [3H]glycerol, which more accurately traces the fate of TG after its lipolysis in LDs, and resynthesis and secretion in lipoproteins. Cells were incubated with [3H]glycerol and 400 μM oleate/BSA to stimulate chylomicron secretion, and radiolabeled TG was quantified in cells and basolateral medium at the end of the labeling period (12 h). In addition, the mobilization and secretion of [3H]glycerol-labeled PC was unaffected in Caco2-KO cells, but secretion was reduced at the end of the incubation period (0 h) (Fig. 8B). During the chase, PC secretion from Caco2-KO cells was similar to controls. The secretion of [3H]glycerol-labeled PE was also reduced in Caco2-KO cells, but intracellular and secreted PE increased slightly during the chase period relative to control cells (Fig. 8C).

Lastly, we monitored the effect of CCTα knockout on the secretion of VLDL and chylomicrons containing apoB100 and apoB48, respectively. CCTα expression in Caco2 cells did not change during differentiation or in response to oleate/BSA addition to the apical medium (Fig. 9A). To determine whether CCTα knockout affected lipoprotein secretion, the 1.006 mg/ml lipoprotein fraction was isolated from the basolateral medium of cells after culturing in the absence or presence of oleate/BSA. Immunoblotting...

Fig. 5. Lyso-PC treatment restores LD size and number in CCTα knockout cells. A: Caco2 and Caco2-KO cells were cultured in oleate/BSA (400 μM) supplemented without (NA, no addition) or with 18:0- or 18:1-lyso-PC (LPC, 25 μM) for 12 h. Cells were immunostained for LMNA/C and LDs were visualized with BODIPY-493/503. B: LD cross-sectional area in Caco2 and Caco2-KO cells was quantified using particle analysis features of ImageJ (v1.48) and binned into area ranges. The results are displayed as box and whisker plots showing the mean and 5th to 95th percentile for six fields of cells from three experiments. *P < 0.005 compared with Caco2 control. C: The number of LDs in each cell was quantified from data in B. **P < 0.01, *P < 0.05.

Fig. 6. Altered LD size and number in differentiated Caco2-KO cells. A: TG mass in cells cultured in the absence (NA, no addition) or presence of oleate/BSA (400 μM) for 12 h. B: Caco2 and Caco2-KO cells were incubated with no addition, 400 μM oleate/BSA, or oleate/BSA plus 18:1-lyso-PC (LPC; 25 μM) for 12 h (OA, oleate/BSA). LDs were visualized by incubation with BODIPY-493/503 and nuclei were immunostained with LMNA/C-monomoclonal and AlexaFluor-594-secondary antibodies. Confocal images are representative of three separate experiments. C: The size distribution of LDs was quantified using particle analysis features of ImageJ (v1.48) and binned into area ranges. The results are box and whisker plots showing the mean and 5th to 95th percentile for six fields of cells from three experiments. ANOVA *P < 0.01, **P < 0.001. D: The number of LDs in each cell was quantified from data in D. *P < 0.05 compared with Caco2 controls. ANOVA *P < 0.01, **P < 0.001.
revealed that apoB48 secretion from Caco2 cells was increased >3-fold in the presence of oleate/BSA (Fig. 9B, C). In contrast, secretion of apoB48 by Caco2-KO cells cultured in the presence or absence of oleate/BSA was <10% of control values (Fig. 9C). Caco2 cells also secreted significant amounts of apoB100 (Fig. 9B). Unlike apoB48, secretion of apoB100 into the 1.006 g/ml lipoprotein fraction by Caco2 cells was not significantly affected by CCTα knockout or the presence of oleate/BSA in the apical medium (Fig. 9D). As well, secretion of apoB100 into the higher density 1.006–1.21 g/ml fraction was unaffected (Fig. 9B). To determine whether 18:1-lyso-PC supplementation could restore apoB48 secretion, cells were cultured in the absence or presence of oleate/BSA and 18:1-lyso-PC for 12 h. However, the secretion of apoB48 by Caco2-KO cells was not restored by exogenous 18:1-lyso-PC (Fig. 9E). Collectively this shows that the secretion of TG and apoB48 in chylomicrons is dependent on the CCTα-catalyzed arm of the CDP-choline pathway.

DISCUSSION

Fatty acids absorbed at the apical surface of human and murine enterocytes are esterified to form TG that is stored in cellular LDs or packaged into chylomicrons for secretion (36, 37). The assembly of LDs and chylomicrons in enterocytes requires a phospholipid monolayer composed primarily of PC, but it is unclear which biosynthetic pathway(s) produce the PC that fulfills this essential function. We utilized Caco2 cells with a knockout of the rate-limiting enzyme in the CDP-choline pathway to show that de novo synthesis of PC is required for TG storage in LDs and the mobilization of TG for secretion in chylomicrons.

In Caco2 cells, the rate-limiting step in the CDP-choline pathway is catalyzed by CCTα and CCTβ2 isoforms. CCTβ2 lacks a nuclear localization signal and is thus restricted to the cytoplasm. As reported for other cultured mammalian cells and tissues (9, 38), CCTα protein was exclusively localized to the nucleoplasm of Caco2 cells, with no evidence of translocation to LDs during oleate stimulation. However,
Phosphatidylcholine (PC) synthesis in liver and kidney is regulated by the CDP-choline pathway, which provides the substrate for cytidine diphosphate (CDP)-choline, a key intermediate in PC biosynthesis. In liver and kidney, the nuclear arm of the CDP-choline pathway is essential for maintaining PC homeostasis. The CDP-choline pathway is also involved in lipid metabolism, with its activity being influenced by the expression of key regulatory proteins such as the cytosolic isoenzyme of cholinephosphotransferase (CCTα).

Metabolic labeling experiments revealed that Metabolic labeling with [3H]choline showed that ER, resulting in a 50% reduction in PC synthesis (Fig. 2). A reduction in PC synthesis is consistent with the knockdown results observed in murine hepatocytes. (15) However, in differentiated Caco2 cells, CRISPR/Cas9-mediated CCTα knockout resulted in a 90% decrease in apoB48 secretion and apoB100 secretion under basal and oleate-treated conditions, as well as a 50% inhibition of TG secretion (based on radioactive oleate and glycerol incorporation). Interestingly, the secretion of apoB100 in VLDL-like particles was not affected in Caco2-KO cells.

The limitation on PC synthesis and cell proliferation imposed by CCTα knockout did not, however, affect the mass distribution of the major molecular species of PC and PE in undifferentiated or differentiated Caco2 cells. Interestingly, the distribution of PC was shifted to longer-chain more unsaturated species when Caco2 cells were differentiated. Because this molecular species shift was independent of reduced de novo PC synthesis and CCTα knockout, changes in Lands remodeling pathways are the probable cause, either due to alternate fatty acid availability or expression of lipases and LPCATs.

Imaging and metabolic labeling experiments showed that loss of the nuclear arm of the CDP-choline pathway and partial inhibition of PC synthesis increased the mass and storage of TG in fewer large LDs. TG accumulation in Caco2-KO cells could be due to increased synthesis resulting from diversion of diacylglycerol from the CDP-choline pathway. However, this is unlikely because undifferentiated Caco2-KO cells actually displayed a slight reduction in the incorporation of [3H]glycerol into TG. Increased LD size is observed in other cell models of PC deficiency, and is attributed to the reduced surface to volume ratio of large LDs and the propensity of small LDs to fuse (41). Similarly, fusion of small nascent LDs and/or reduced turnover of TG in large LDs could explain the abnormal TG storage phenotype of Caco-2-KO cells. The observation that 18:1-lyso-PC more effectively reversed LD morphology compared with 18:0-lyso-PC, which produced smaller droplets than those containing saturated PC, suggests that the source of PC is not critical for the maintenance of LD morphology. Supplementation with 18:1-lyso-PC more effectively reversed LD morphology compared with 18:0-lyso-PC. This is consistent with a study using artificial emulsions of TG and unsaturated PC, which produced smaller droplets than those containing saturated PC (42). The reduced efficacy of 18:0-LPC that we observed in Caco2 cells could also be due to reduced cellular uptake or utilization by LPCATs.

Differentiated Caco2 cells primarily secrete apo100-containing VLDL-like particles under basal conditions, but incubation with exogenous fatty acids stimulates the secretion of apoB48-containing chylomicrons (31). Similarly, we observed a 3-fold increase in secretion of apoB48-containing chylomicrons when oleate was added to the apical medium. CCTα knockout resulted in a 90% decrease in apoB48 secretion under basal and oleate-treated conditions, as well as a 50% inhibition of TG secretion (based on radioactive oleate and glycerol incorporation). Interestingly, the secretion of apoB100 in VLDL-like particles was not affected in Caco2-KO cells. The lack of effect on apoB100 secretion was unexpected because CCTα knockout in murine hepatocytes resulted in a 75% reduction in PC synthesis and inhibition

Fig. 9. CCTα knockout decreases apoB48 chylomicron secretion by differentiated Caco2 cells. A: CCTα expression was monitored in Caco2 cells during a 21 day differentiation period and after exposure to oleate/BSA for 12 h. B: Differentiated Caco2 and Caco2-KO cells were treated with oleate/BSA (400 μM) on the apical surface for 12 h. Basolateral medium was collected and lipoproteins in the 1.006 g/ml and 1.006–1.21 g/ml density fractions were isolated by sequential ultracentrifugation. apoB48 and apoB100 was resolved by SDS-5%PAGE and immunoblotting. D: apoB48 (C) and apoB100 (D) in the d = 1.006 g/ml fraction was quantified from immunoblots in B and expressed relative to cellular actin. The results are the mean and SEM of three experiments. *P < 0.01, **P < 0.005 compared with Caco2 controls. E: Cells were treated with the indicated combinations of oleate/BSA (400 μM) and 18:1-lyso-PC (25 μM) in apical medium for 12 h. Lipoproteins in the 1.006 g/ml fraction were isolated and immunoblotted for apoB as described above.

adult human intestine expresses only the CCTα isoform (39). Because Caco2 cells have features of fetal tissue, such as the expression of both apoB48 and apoB100 (40), the expression of CCTβ likely reflects the differentiation stage of this epithelial carcinoma. Indeed, expression of CCTβ mRNA in some fetal tissues typically decreases or disappears during development (15). Due to expression of both CCT isoforms in Caco2 cells, CRISPR/Cas9-mediated knockout of CCTα produced cells in which all steps of the CDP-choline pathway were restricted to the cytoplasm and ER, resulting in an approximate 50% reduction in PC synthesis. Metabolic labeling with [3H]choline showed that loss of nuclear CCTα in Caco2 cells did not cause an acute bottleneck in the pathway because the substrate, [3H]pCholine, did not accumulate, but there was a transient decrease in the product, [3H]CDP-choline (Fig. 2). A reduction in PC synthesis in Caco2-KO cells did not lead to a reciprocal slowdown in degradation to maintain PC levels. Rather, the proliferation of knockout cells was reduced by 30–40% because residual CDP-choline pathway activity provided by cytoplasmic CCTβ2 was insufficient.
of apoB100/VLDL secretion (22, 43). The insensitivity of apoB100 secretion to CCTα knockout in Caco2 cells could be due to less severe inhibition of the CDP-choline pathway due to remaining CCTβ2 expression and/or a reduced requirement for phospholipids and TG to assemble VLDL. In contrast, the secretion of TG-rich apoB48/chylomicrons was highly sensitive to ablation of nuclear CCTα and partial disruption of the CDP-choline pathway.

The blockage of chylomicron and TG secretion in differentiated Caco2-KO cells was also accompanied by fewer and larger LDs and accumulation of intracellular TG. However, the storage of TG in Caco2-KO cells was not due to inhibition of chylomicron secretion because secreted TG (based on oleate- or glycerol-labeling) constituted only a small fraction of the cellular pool (2–4%) and would minimally impact cellular accumulation. An existing pool of LDs in Caco2 cells, and their abnormal size and distribution in the knockout cells, could explain the differential effect on apoB100 and apoB48 secretion. The secretion of VLDL TG is not stimulated by oleate and utilizes preformed TG (35), which could be supplied by preexisting LD stores in Caco2 cells. This contrasts with TG secreted in chylomicrons, which is primarily derived from de novo synthesis (35). Thus, in Caco2-KO cells cultured in basal medium, a cellular pool of TG would be available for constitutive secretion of apoB100/VLDL, but not apoB48/chylomicrons. Addition of oleate/BSA to control or Caco2-KO cells increased the cellular TG stores; however, de novo synthesized TG was not available for secretion of chylomicrons by Caco2-KO cells due to more efficient packaging in LDs and/or defective assembly into chylomicrons. It is uncertain whether the TG secreted in chylomicrons is initially packaged in LDs and then mobilized by lipolysis, or is directly incorporated in the ER. The reduced secretion of [1H]glycerol-labeled TG by Caco2-KO cells during a chase period suggests that mobilization of TG in LDs is partially defective.

Because 18:1-lyso-PC supplementation of Caco2-KO cells partially restored LD morphology (Figs. 5, 6), we tested to determine whether apoB48 secretion was enhanced. However, 18:1-lyso-PC had no effect on apoB48 or apoB100 secretion by either Caco2 or Caco2-KO cells. It is possible that exogenous 18:1-lyso-PC is preferentially acylated by LPCAT to form PC on the surface of LDs (7, 34). LPCAT activity for assembly of chylomicrons could still be limiting due to insufficient PC synthesis in the ER by the terminal enzyme in CDP-choline pathway. This suggests redundancy between the Lands and CDP-choline pathways for PC incorporation to LDs, but that the latter pathway is the preferred source of PC for chylomicron assembly in the ER [1A].

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