Hepatic CTP:Phosphocholine Cytidyltransferase-α Is a Critical Predictor of Plasma High Density Lipoprotein and Very Low Density Lipoprotein*

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CTP:phosphocholine cytidyltransferase (CT) is the key regulatory enzyme in the CDP-choline pathway for the biosynthesis of phosphatidylcholine (PC). We previously generated a mouse in which the hepatic CTα gene was specifically inactivated by the cre/loxP procedure. In CTα knock-out mice, plasma high density lipoprotein (HDL) and very low density lipoprotein (VLDL) levels were markedly lower than in wild type mice (Jacobs, R. L., Devlin, C., Tabas, I., and Vance, D. E. (2004) J. Biol. Chem. 279, 47402–47410). To investigate the mechanism(s) responsible for the decrease in plasma lipoprotein levels, we isolated primary hepatocytes from knock-out and wild type mice. ABCA1 expression was reduced in knock-out hepatocytes and apoAI-dependent cholesterol, and PC efflux was impaired. When knock-out hepatocytes were infected with an adenovirus expressing CTα, apoAI-dependent PC efflux returned partially, whereas cholesterol efflux and ABCA1 levels were not restored to normal levels. Adenoviral expression of CTα did not increase VLDL secretion in knock-out hepatocytes, even though cellular PC levels returned to normal. However, in vivo adenoviral delivery of CTα normalized plasma HDL and VLDL levels in knock-out mice. The observations demonstrate that hepatic PC biosynthesis is a key player in maintaining plasma VLDL and HDL, and further underscores the importance of the liver in HDL formation.

In mammals, phosphatidylcholine (PC)4 is the primary phospholipid in cellular membranes, bile, lung surfactant, and plasma lipoproteins. In all nucleated tissues, PC is made via the Kennedy (CDP-choline) pathway (1), and the activity of CTP:phosphocholine cytidyltransferase (CT) usually regulates the flux through this pathway (2–4). In mice, as in humans, two genes named Pcyt1a and Pcyt1b, encode CT (5). The gene product of Pcyt1a, CTα, is believed to be the predominant isoform in liver (6, 7). PC is also synthesized in the liver via the sequential methylation of phosphatidylethanolamine (PE) catalyzed by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT) (8). Several studies have indicated that ~30% of the PC in liver is made via the PEMT pathway, whereas the remainder is synthesized via the CDP-choline pathway (9–11).

The function of both PC biosynthetic pathways has been a topic of interest in our laboratory for many years. Recently, we generated mice deficient in either PEMT or hepatic CTα, allowing us to gain insight into the role of these pathways in lipid metabolism. Pemt−/− mice appear normal when fed a chow diet, suggesting that the CDP-choline pathway is sufficient for life, providing that dietary choline is available (12–14). However, a specific role for PEMT in VLDL secretion has been demonstrated in vivo and in hepatocyte experiments (15, 16). In male Pemt−/− mice fed a high fat/cholesterol diet, plasma triacylglycerol (TG) and PC levels were decreased compared with controls (16). In Pemt−/−/hepatocytes, apoB100 and TG secretion was reduced by 60 and 75%, respectively (16). This reduction in VLDL secretion in the Pemt−/− mice occurred even though hepatic CT activity was increased nearly 2-fold (17). Furthermore, hepatic PC mass was the same in Pemt−/− mice and wild type littermates (16). These studies demonstrate that CT does not compensate for the lack of PEMT activity. Thus it appears that PEMT-derived PC is important in maintaining VLDL secretion from hepatocytes.

The function of CTα has been investigated in several genetically modified mouse models. Global loss of CTα expression is lethal at embryonic day E3.5 (18). Tissue-specific deletion of CTα has been performed in macrophages (19), liver (6, 7), and lung (20). Jackowski and co-workers (20) have shown that CTα is not essential for proliferation or differentiation of lung epithelia. However, pulmonary CTα is essential for the synthesis and secretion of PC in lung surfactant. Without normal PC secretion, lungs fail to inflate at birth, accumulate TG, and have abnormal lamellar bodies. Similarly, CTα is not essential in the development of macrophages but is necessary to protect against cholesterol-induced cell death (19).
Decreased Lipid Secretion from CTα-deficient Hepatocytes

We previously reported that mice lacking hepatic CTα are viable and fertile. However, as a result of impaired CT activity, the mass of PC in the liver is reduced by 6–25%, whereas TG accumulates (6). Furthermore, the levels of plasma lipoprotein cholesterol, TG, and PC are markedly reduced. The reduction of plasma TG is likely due to decreased secretion of VLDL from the liver (6).

In contrast to VLDL secretion, the link between hepatic PC production and high density lipoprotein (HDL) metabolism has not been studied thoroughly. Unlike VLDL, choline deficiency does not reduce plasma HDL levels in rats (21). Furthermore, the amount of PC, cholesterol, or apoAI in the medium from hepatocytes incubated with choline- and methionine-deficient medium is not reduced (22). It was therefore unexpected that both the Pemt<sup>−/−</sup> mice and the liver-specific CTα knock-out mice had lower HDL than their respective wild type littersmates (6, 16). Moreover, liver-specific deletion of the ATP-binding cassette transporter 1 (ABCA1), which mobilizes both cellular phospholipids and cholesterol to lipid-poor apoAI, reduces plasma HDL-cholesterol by 80% (23). We considered the possibility that impairment of PC biosynthesis reduces the amount of PC available for lipidation of apoAI and results in decreased levels of plasma HDL. Thus, using CTα-deficient hepatocytes as a model, we investigated whether or not impaired PC biosynthesis decreases HDL formation. We predicted that both VLDL secretion and HDL formation would be impaired in CTα-deficient hepatocytes. We also hypothesized that if CT activity were re-established, via adenoviral gene delivery, ample PC would be available to normalize both VLDL and HDL formation.

EXPERIMENTAL PROCEDURES

Materials—Sheep anti-human apoB antibody was purchased from Roche Applied Science, and rabbit anti-human apoAI antibody was from Biodesigns (Kennebunk, ME). Anti-goat hemagglutinin (HA) and anti-rabbit lamin A/C antibodies were purchased from Sigma and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Texas Red-X goat anti-mouse and Alexa Fluor 488 goat anti-rabbit antibodies were purchased from Molecular Probes (Eugene, OR). Donkey anti-sheep antibodies and goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase were purchased from Pierce. All other chemicals and reagents were from standard commercial sources. Anti-ABCA1 antibody was purchased from Novus Biologicals, Inc. (Littleton, CO).

Animal Care—All procedures were approved by the Institutional Animal Care Committee, University of Alberta, and were in accordance with guidelines of the Canadian Council on Animal Care. Female mice (3–6 months old) were fed ad libitum a chow diet (PicoLab Rodent Diet 20) or a semi-purified diet in Dulbecco’s modified Eagle’s medium (DMEM) containing 15% fetal bovine serum (FBS). Two h after plating, the cultures were rinsed twice in serum-free DMEM over a 1-h period. For experiments on VLDL secretion, hepatocytes were incubated in serum-free DMEM for 10–16 h. Medium was concentrated using Amicon® ultracentrifugal filter devices (Millipore).

Lipid Efflux from Hepatocytes—Hepatocytes were washed twice with FBS-free DMEM and then incubated with FBS-free DMEM containing [3H]mevalonate (10 μCi/ml) and [3H]choline (5 μCi/ml) for 4 h. The cells were then washed twice with cold FBS-free DMEM and incubated with FBS-free DMEM with or without exogenous apoAI (10 μg/ml) for 3–9 h. Lipids from cells and medium were extracted (24) and separated by thin layer chromatography, and the radiolabel associated with PC and cholesterol was measured with a scintillation counter (25). Lipid efflux was calculated as a percentage of counts in the media divided by the total of the counts in cells and media.

Adenoviral Infection of Hepatocytes—To construct recombinant adenovirus, a cDNA encoding an HA-tagged CTα was subcloned into a pAdTrack-CMV shuttle vector, linearized with Pmel, and inserted into adenovirus using pAdEasy-1 system for homologous recombination in Escherichia coli (26). The pAdTrack-CMV shuttle vector also contained a gene encoding green fluorescent protein (GFP). Therefore, the adenovirus used to express CTα protein also expressed GFP, which served as a marker of successful viral infection and protein expression. Fluorescent titering was used to estimate the concentration of each adenovirus preparation. In some experiments, hepatocytes were infected with Ad.GFP or Ad.ΔCTα (2 plaque-forming units/cell) for 16 h before the start of the experiment.

Confocal Microscopy—COS cells were seeded onto 22-mm coverslips in 6-well plates at a density of 0.5 × 10<sup>5</sup> cells/well. Following overnight incubation at 37 °C, 5% CO<sub>2</sub>, the cells were transfected with pCI plasmids encoding HA-tagged CTα. Twenty four h post-transfection, cells were serum-starved for 1 h and fixed in 4% paraformaldehyde/phosphate-buffered saline, pH 7, for 10 min. Following permeabilization with 0.2% Triton X-100/phosphate-buffered saline for 2 min, cells were incubated with the first primary antibody (anti-lamin A/C, 1:100) at 37 °C for 1 h. The cells were then incubated with the second primary antibody (monoclonal anti-HA, 1:150) for 1 h at 37 °C. Cells were subsequently incubated for 1 h at 37 °C with both secondary antibodies, Texas Red-X goat anti-mouse (1:200) and Alexa Fluor 488 goat anti-rabbit (1:200). Coverslips were mounted onto slides with Prolong Antifade™ reagent, and immunofluorescence was visualized using a Zeiss LSM510 confocal microscope.

McArdle RH-7777 Cell Culture and Stable Transfection—McArdle cells were maintained in DMEM supplemented with 10% (v/v) FBS and 10% (v/v) horse serum at 37 °C in a 5% CO₂ incubator. The cells were transfected by the calcium phosphate precipitation method with either pcDNA3.1-CTα or pcDNA3.1 vector, which encodes for neomycin resistance. The cDNA for rat CTα without an HA tag was used in these experiments. Stable colonies were selected in culture medium containing 400 μg/ml G418 and maintained in medium containing 200 μg/ml G418. Triplicate 60-mm dishes of cells (1 × 10<sup>6</sup> cells/dish) were seeded 20 h before the start of the experiment. The cells were incubated in serum-free DMEM containing 0.4 mM oleate/bovine serum albumin for 30 min. The medium was replaced with DMEM that contained 10 μCi of [3H]glycerol in the presence of...
0.4 mM oleate/bovine serum albumin for 4 h. The lipids were extracted and fractionated by thin layer chromatography, and radioactivity was determined.

Injection of Recombinant Adenoviruses—A single dose of Ad.GFP or Ad.CT (1.0 \times 10^9 plaque-forming units) was injected into female wild type or knock-out mice via the tail vein. Seven days after injection, the mice were fasted (16 h) and anesthetized with metofane. A blood sample was drawn and plasma retained. Livers were dissected immediately and stored at −80 °C for further analysis.

Immunoblot Analyses—Homogenates (40 μg of protein) or concentrated medium was boiled in buffer containing 1% SDS, and proteins were separated on SDS-polyacrylamide gel. Proteins were transferred to an Immobilon-P transfer membrane and probed with anti-GFP (dilution 1:5000), anti-HA (dilution 1:10000), anti-ABC1 (dilution 1:1000), anti-apoAI (dilution 1:10,000), anti-albumin (dilution 1:5000), or anti-apoB (dilution 1:5000) antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences).

Determination of Lipid Mass—The amount of cholesterol, cholesteryl ester, and TG was determined in liver/cell homogenates (0.5 mg of protein), hepatocyte-conditioned medium (3 ml), and plasma (100 μl). After digestion (2 h, 30 °C) of the phospholipids with phospholipase C, tridecanoin (20 ng) was added as an internal standard, and lipids were extracted. The mass of TG, cholesterol, and cholesteryl ester was determined by gas-liquid chromatography (27). Phospholipids were separated by thin layer chromatography, and the mass of PC and PE was determined via a lipid phosphorus assay (28).

For visualization of lipid droplets, hepatocytes were plated onto 24-well plates at a density of 0.5 \times 10^4 cells/well. After 1 h of incubation in serum-free medium, cells were fixed with 4% paraformaldehyde/phosphate-buffered saline, pH 7.0, for 10 min at room temperature. Following three washes with phosphate-buffered saline, cells were incubated with 2 μg/ml BODIPY (Molecular Probes, number 3922) for 20 min, washed three more times with phosphate-buffered saline, and images acquired using a fluorescence microscope.

Analysis of Plasma and Medium Lipoprotein Lipid Profiles—Plasma or hepatocyte-conditioned medium was separated into lipoprotein fractions using high performance liquid chromatography with an Amersham Biosciences Superose 6 column attached to a Beckman Systems Gold or Nouveau Gold apparatus. In-line assays for total cholesterol (Sigma Infinity cholesterol reagent), TG (Sigma triglyceride GPO Trinder kit), and PC (Sigma phospholipids B kit) were performed as described previously (6).

Statistical Analysis—Data are presented as means ± S.D. unless otherwise noted. Each experimental group contained three to four samples. Analysis of variance was performed to compare means unless otherwise specified. A p value of <0.05 was considered to be significant.

RESULTS

VLDL Secretion Is Impaired in CTα-deficient Hepatocytes—Hepatocytes isolated from wild type and hepatic CTα knock-out mice were incubated in serum-free DMEM for 16 h. As shown in Fig. 1A, CTα-deficient hepatocytes secrete ~50% less PC and TG into the medium compared with wild type cells. Furthermore, apoB48 secretion was reduced by ~50% in knock-out hepatocytes, whereas apoB100 secretion was almost completely abolished (Fig. 1B). Diminished VLDL secretion from CTα-deficient cells correlated with a 2-fold increase in hepatocyte TG (Fig. 1C), which accumulated in large lipid droplets (Fig. 1D). Interestingly, large lipid droplets were predominantly perinuclear compared with lipid droplets in control cells, which accumulated more in the cell periphery. The decrease in VLDL secretion and accumulation of intracellular TG in knock-out cells may have been a result of altered phospholipid concentration because cellular PC levels were reduced by 30% (Fig. 1C).

ABCA1-mediated Lipid Efflux Is Decreased in CTα-deficient Hepatocytes—We previously reported that plasma HDL (cholesterol and apoAI) levels were reduced in the liver-specific CTα knock-out mouse (6). To evaluate the role of hepatic CTα in HDL formation, we incubated wild type and knock-out hepatocytes in the presence or absence of apoAI. As shown in Fig. 2, A and B, the efflux of [3H]PC and [3H]cholesterol from wild type and knock-out hepatocytes was linear for at least 9 h. The release of [3H]PC and [3H]cholesterol from wild type hepatocytes was stimulated by ~2- and 3-fold by apoAI, respectively. In knock-out cells, apoAI induced a 2-fold increase of [3H]cholesterol secretion into the medium but did not increase the amount of [3H]PC released. Therefore, knock-out hepatocytes effluxed less lipids to apoAI compared with control cells. The mass of cholesterol in the culture medium was also determined after 10 h of incubation and confirmed that knock-out hepatocytes had impaired apoAI-induced cholesterol efflux (Fig. 2E).

Endogenous apoAI secretion was not altered by genotype (Fig. 1B), and the lipid efflux experiments showed that apoAI...
was lipidated normally (Fig. 2, A–C). Fast protein liquid chromatography analysis of the lipoproteins secreted by cells incubated with exogenous apoAI recapitulated the impairment in HDL formation in the knock-out mice (Fig. 2D). One surprising result was that the size of HDL was larger in the medium taken from knock-out hepatocytes compared with controls (Fig. 2D). This result is consistent, however, with the results in Fig. 2E, which show that [3H]cholesterol efflux was stimulated in knock-out hepatocytes by apoAI without increased efflux of [3H]PC.

The membrane protein ABCA1 plays a critical role in HDL formation by facilitating the efflux of cellular lipids to an apolipoprotein acceptor (29). With this in mind, hepatocytes were homogenized and the levels of ABCA1 protein evaluated (Fig. 2F). In agreement with the efflux assay, ABCA1 protein levels were reduced by ~50% in CTα-deficient hepatocytes.

Adenoviral Expression of CTα Normalizes PC Levels in Knock-out Hepatocytes—To test whether the impairment of lipid secretion was reversible in knock-out hepatocytes, we created a recombinant adenovirus that expresses an HA-tagged CTα cDNA. A pCI expression vector containing the tagged CTα cDNA was transiently transfected into COS cells, and the expressed enzyme was shown to have in vitro activity and to stimulate [3H]choline incorporation into PC (data not shown). Furthermore, the protein was properly located within the nucleus (Fig. 3A). When hepatocytes were infected with the adenovirus, the expected bands for CTα and/or GFP were detected by immunoblotting (Fig. 3B). Adenoviral expression of CTα in wild type hepatocytes resulted in a 2-fold increase in incorporation of radioactivity in PC from either [3H]choline (Fig. 3C) or [3H]glycerol (Fig. 3D). As reported previously (6), choline-dependent PC biosynthesis was reduced by 85% in CTα-deficient hepatocytes, whereas total PC production was reduced by 60%. Adenoviral CTα infection of knock-out cells resulted in a 10- and 5-fold increase in [3H]choline and [3H]glycerol incorporation into PC, respectively. Therefore, after adenoviral infection the apparent rate of hepatic PC biosynthesis was equal in knock-out and wild type cells.

The mass of PC was 30% lower in knock-out hepatocytes compared with control cells (Fig. 3E). Ad.CTα infection restored PC mass to control levels without altering the mass of PE (Fig. 3F). Interestingly, PC mass in wild type cells was not increased following adenoviral infection, suggesting increased turnover of newly formed PC in these cells. Indeed, following an 8-h chase, the disappearance of radiolabeled PC was greater in
control cells infected with Ad.CT (38 ± 6%) versus those treated with Ad.GFP (8 ± 5%, p < 0.05). However, the turnover of PC in knock-out cells was not significantly increased by CT overexpression (PC disappearance (% initial): Ad.GFP = 9 ± 6% versus Ad.CT = 17 ± 10%, p = 0.32).

Adenoviral Expression of CTα Partially Restores Lipid Efflux to ApoAI in Knock-out Hepatocytes—To investigate whether or not lipid efflux was altered by adenoviral expression of CTα, we incubated hepatocytes with or without apoAI following Ad.GFP or Ad.CTα infection. Overexpression of CTα in wild type hepatocytes did not alter the mass of PC or cholesterol effluxed to apoAI (Fig. 4, A and B). However, when Ad.CTα-infected knock-out cells were treated with apoAI, the amount of PC in the medium increased by 30% compared with that in Ad.GFP-treated hepatocytes, although PC efflux remained lower than that in wild type cells. Similar experiments measuring the efflux of [3H]cholesterol and [3H]PC were performed (Fig. 4, C and D). Ad.CTα infection of knock-out hepatocytes completely normalized PC efflux to apoAI (Fig. 4C). The size of the HDL in the medium of knock-out cells was also partially returned to normal (Fig. 4E). Interestingly, however, cholesterol efflux to apoAI was not significantly increased following Ad.CTα, which suggests that restoring the total amount of cellular PC was not sufficient to restore HDL formation in knock-out hepatocytes.

To understand why HDL formation was not completely normalized by adenoviral expression of CTα, we evaluated the levels of cellular ABCA1. Although PC levels returned to normal following adenoviral induced overexpression of CTα, ABCA1 protein levels did not increase in knock-out hepatocytes (Fig. 4F) incubated with Ad.CTα. ABCA1 was also not changed in wild type cells expressing adenoviral CTα.

Adenoviral Expression of CTα Does Not Increase VLDL Secretion from Hepatocytes—Many studies have shown a link between reduced hepatic PC biosynthesis and impaired VLDL-TG secretion (15, 16, 21, 22). Therefore, we predicted that adenoviral expression of CTα, which normalized cellular PC mass, would increase VLDL secretion in knock-out hepatocytes. However, the amount of VLDL-TG secreted from knock-out cells was not altered by Ad.CTα infection (Fig. 5, A and B). Unexpectedly, overexpression of CTα in wild type cells, which did not alter cellular PC levels, resulted in a 50% reduction in TG secretion (Fig. 5B). Infection of cells with Ad.CTα did not alter cellular TG or cholesterol levels in either control or knock-out hepatocytes (data not shown).

Because it appears that overexpression of CTα in normal cells causes a defect in VLDL secretion, we reasoned that over-
expression of CTα in McArdle rat hepatoma cells would provide the same result. Therefore, we transfected McArdle cells with a cDNA encoding CTα under control of the cytomegalovirus promoter. Three stable cell lines, with low (2×), medium (10×), and high (20×) CTα overexpression (data not shown) were selected to study VLDL secretion. The vector-transfected cells, containing endogenous CT activity, were used as a control. When incubated with [3H]glycerol for 2 h, transfected cells synthesized 40% more [3H]PC, whereas [3H]TG production was not altered (data not shown). The enhanced PC synthesis was independent of the level of CTα activity. Although McArdle cells produced more intracellular PC, they secreted 20–50% less than cells transfected with vector alone (data not shown). TG secretion was also reduced (30–60%) in cells overexpressing CTα (data not shown). This result is consistent with our data in primary hepatocytes and illuminates an inhibitory role of high levels of CT on VLDL secretion.

In Vivo Expression of CTα Normalizes Hepatic PC Mass and Plasma Lipoprotein Levels in Knock-out Mice—To determine whether in vivo restoration of hepatic CT activity would normalize plasma lipoprotein levels in the knock-out mice, we injected mice with either Ad.GFP or Ad.CTα via the tail vein. After 7 days, liver and plasma samples were isolated and analyzed. As reported previously (6), total hepatic CT activity in knock-out mice was reduced to ∼15% of control levels (Fig. 6A). Following Ad.CTα injection, total CT activity was restored to wild type levels. The active form of CT in cells is membrane-bound, whereas the soluble form of CT is thought to be an inactive reservoir. In vitro activity in both membrane and cytosolic fractions was normalized in knock-out mice following Ad.CTα administration (Fig. 6A).

Steady-state levels of hepatic PC were moderately (20%) reduced in knock-out mice compared with controls (Fig. 6B). Ad.CTα administration restored PC mass in knock-out livers to normal. Similar to our hepatocyte experiments, the concentration of PE was not altered by genotype or adenoviral treatment. Plasma cholesterol was decreased in all lipoprotein fractions in knock-out mice (Fig. 7A). Restoration of CT activity in knock-out mice normalized cholesterol in HDL and VLDL fractions but not in low density lipoproteins. Hepatic CT activity strongly correlated (R² = 0.91) with total plasma cholesterol (Fig. 7B). In contrast to hepatocytes, in vivo Ad.CTα administration to knock-out mice increased ABCA1 levels (Fig. 7C).

Plasma VLDL-TG was reduced by 70% in knock-out mice as compared with controls (Fig. 8A) and was returned to normal levels following Ad.CTα injection. Furthermore, total plasma TG levels positively correlated (R² = 0.45) with hepatic CTα activity (Fig. 8B).

DISCUSSION

We sought to explain why plasma VLDL and HDL levels are reduced in liver-specific CTα knock-out mice. The experiments presented herein demonstrate that VLDL secretion and HDL formation are compromised in murine hepatocytes deficient in CTα. Whereas forced expression of CT by adenoviral delivery reversed the PC deficit in hepatocytes, lipid secretion was not normalized. However, in vivo restoration of hepatic PC
biosynthesis did restore lipoprotein levels, suggesting that normalization of plasma lipoproteins cannot be attributed solely to normalization of hepatic PC levels.

Why Is VLDL Secretion Impaired in CTα-deficient Hepatocytes?—In 1932, Best and Huntsman (30) illuminated the nutritional importance of choline. Since then, it has been shown that a diet deficient in choline induces hepatic steatosis in mice (31), rats (21), and more recently in humans (32, 33). In rodent experiments, the level of plasma VLDL is reduced by a choline-deficient diet which may, at least partially, account for the hepatic lipid accumulation (33). The role of PC biosynthesis in VLDL secretion from hepatocytes has been studied intently in our laboratory. Hepatocytes incubated with medium deficient in methionine and choline, but not choline alone (34), had impaired secretion of lipids (PC and TG) and apoB48 and apoB100 associated with VLDL (22). Mechanistic studies indicated that choline deficiency in rats results in production of nascent PC-deficient VLDL particles (35). These PC-deficient particles were found to be degraded in a post-endoplasmic reticulum compartment. Subcellular fractionation of choline-deficient livers showed that the PC/PE ratio is significantly reduced in microsomes leading to formation of abnormal VLDL particles (36). Subsequent studies demonstrated that the addition of choline, lyso-PC, or even methionine to the medium of primary rat hepatocytes reversed the block in VLDL secretion (22, 37). This reversal was dependent on the choline moiety, because neither monomethyllethanolamine nor dimethylethanolamine can substitute (38). Furthermore, whereas propanolamine can replace choline in yeast (39, 40), it is unable to do so in mammalian cells (41).

We reported that plasma PC, TG, and apoB100 are decreased in the liver-specific CTα knock-out mouse (6). As predicted, we now show that VLDL secretion is impaired in CTα knock-out hepatocytes. Because impaired VLDL secretion induced by choline deficiency is reversible (22, 37, 38, 41), we predicted that infecting knock-out hepatocytes with Ad.CTα would also normalize lipoprotein secretion. Surprisingly, adenoviral overexpression of CT in knock-out hepatocytes did not increase VLDL secretion, even though PC biosynthesis was increased 2-fold above normal levels. These observations suggest that secondary factors, other than decreased PC biosynthesis, are involved in inhibiting VLDL secretion in knock-out hepatocytes. If this were the case, it is also apparent that “short term” expression of CT did not influence these yet to be determined factors. Our in vivo data support this assertion. As in the hepatocytes, adenoviral expression of CT in knock-out mice increased hepatic PC levels. However, in this “long term” experiment VLDL levels returned to control levels, and the level of plasma TG positively correlated with hepatic CT activity. Thus, it is possible that the rectification of VLDL metabolism requires an extended period after the increase in CTα that was not recapitulated in primary hepatocytes, because of the limited life span of these cells in culture. Another potential difference between the in vivo and hepatocyte experiments is the level of CT activity induced by adenoviral infection. Total CT activity returned to normal following tail vein injection of Ad.CT, whereas inclusion of the virus in hepatocyte culture medium resulted in a 20-fold increase in CT activity (data not shown). As outlined below, it is possible that super-physiological levels of CT impair VLDL secretion from hepatocytes.

The ability of CT overexpression to inhibit TG secretion in wild type primary hepatocytes and cultured hepatoma cells was surprising and was particularly perplexing because VLDL secretion is increased in McArdle cells transfected with PEMT (15). When CTα was transfected into COS cells, CT activity was increased by ~20–100-fold, whereas [3H]choline and [3H]glycerol incorporation into PC was elevated only 3-fold, and cellular PC was only marginally increased because of a 3-fold increase in PC degradation rate (42). Furthermore, overexpression of CT did not alter PC levels in Chinese hamster ovary cells (43). These studies could provide an explanation for our observations. It is plausible that an increased rate of PC biosynthesis and degradation creates a highly active futile cycle, which prevents newly formed PC from being used in packaging and secretion of VLDL.

ABCA1-dependent Lipid Efflux Is Decreased in CTα-deficient Hepatocytes—Plasma HDL is inversely correlated with the development of cardiovascular disease. HDL is postulated to impair atherogenesis by stimulating transport of cholesterol from peripheral cells, such as macrophages, to the liver (44–46). The ABCA1 transporter controls the influx of both cellular phospholipids and cholesterol to lipid-poor apoAI (47, 48). The
liver has high expression of both ABCA1 and apoAI and has been proposed to be the most significant source of plasma HDL (49–51). We have previously reported that the liver-specific CTα knock-out mouse displays a 40–60% reduction in plasma cholesterol, PC, and apoAI (6). These findings led us to hypothesize that impaired hepatic PC biosynthesis influences ABCA1-mediated lipid efflux. Our results clearly show that cholesterol and PC efflux to apoAI is impaired by CTα deficiency in the hepatocytes. Moreover, hepatic ABCA1 is decreased by 50% in knock-out hepatocytes without alterations in cellular cholesterol, which has been identified as an important regulator of ABCA1 expression (52). However, PC levels were decreased by 30% in knock-out cells, suggesting a link between cellular PC availability and ABCA1 expression and activity. Our data are consistent with that of Zhou et al. (53) who showed that overexpression of CT increases ABCA1-mediated PC efflux in lung cell cultures. The level of ABCA1 has also been linked to the fatty acid side chains of PC. Recently, Wang and Oram (54, 55) showed that when unsaturated fatty acids, such as arachidonate, are incorporated into phospholipids, the stability of ABCA1 is decreased through a phospholipase D/protein kinase Cδ-dependent mechanism. This is germane to our study because the level of arachidonate in PC was increased 2-fold in CTα-deficient livers as compared with controls (data not shown). Thus we can speculate that both the amount and species of PC may be involved in regulating ABCA1 activity.

An emerging consensus is that the liver plays a key role in determining plasma HDL levels. Overexpression of hepatic ABCA1 results in a 2-fold increase in plasma HDL (50), whereas targeted deletion of hepatic ABCA1 reduces plasma HDL-cholesterol by 80% (23). In the latter studies, the level of HDL-cholesterol was directly proportional to the expression of hepatic ABCA1. The impairment of hepatic cholesterol and PC efflux resulted in poorly lipidated plasma apoAI, which was quickly catabolized by the kidney. Singaraja et al. (56, 57) confirmed that hepatic ABCA1 is necessary for the formation of nascent HDL, and showed that the liver contributes the majority of the HDL phospholipids. In contrast, the bulk of cholesterol was proposed to be added to HDL by the action of ABCA1 and ABCG1 in peripheral tissues (56).

Although the etiology differs, our results substantiate the view that hepatic ABCA1 is important in determining plasma HDL levels. ApoAI secretion was not decreased in CTα-deficient hepatocytes consistent with findings that choline deficiency does not reduce the secretion of apoAI from hepatocytes (22). Studies in Tangier disease subjects (58) and in Abca1−/− mice demonstrated that ABCA1 is not required for apoAI secretion from hepatocytes (23, 59). Rather, our studies indicate that impaired hepatic PC biosynthesis reduces both ABCA1 protein levels and apoAI lipidation. Interestingly, ABCA1 deficiency has differing effects on PC and cholesterol efflux. For example, in Abca1−/− hepatocytes, apoAI phospholipidation is reduced by 80%, whereas cholesterol efflux is reduced by only 40% (60). Our results in CTα-deficient hepatocytes mirror these findings; apoAI-dependent cholesterol efflux was reduced by 40%, whereas the addition of exogenous apoAI did not stimulate PC secretion. To compensate for the lack of PC availability, the knock-out hepatocytes produced larger, and thus fewer, HDL particles.

To determine whether the altered lipid efflux was because of lack of PC and/or decreased ABCA1 expression, we reintroduced CT activity into hepatocytes and mice using an adenovirus. As predicted, Ad.CTα infection restored PC biosynthesis and PC mass in knock-out cells to control levels. However, the impact of Ad.CTα on lipid efflux was more complicated. First, PC biosynthesis was restored, but apoAI-dependent PC efflux was not normalized. Second, Ad.CTα infection exerted no influence on cholesterol efflux in knock-out hepatocytes. This preferential increase of PC secretion reduced the size of the HDL particles such that they resembled those in wild type medium, consistent with the idea that the lipidation of apoAI by PC and cholesterol is separately regulated (59, 60). Furthermore, these changes in HDL production occurred without altered ABCA1 expression. It is possible that our observations could be the result of altered activities of other members of the ABC transporter family. For example, ABCA7 was shown to selectively efflux phospholipids to apoAI, whereas ABCG1 mediates the efflux of cholesterol to partially lipidated HDL (61–64). However, this seems unlikely because hepatocytes have very low levels of ABCG1 mRNA (62, 63), and in agreement with others (61), we were unable to detect ABCA7 protein in hepatocytes (data not shown). Taken together, our data indicate that both the deficiency of PC and the decreased expression of ABCA1 contribute to the impairment of HDL formation in CTα-deficient hepatocytes.

Our in vivo data strengthen the link between PC biosynthesis and ABCA1 expression. Seven days after Ad.CTα delivery, hepatic ABCA1 levels and plasma HDL were similar to control littermates. One curious difference between the in vivo and hepatocyte experiments was the relative size of the HDL. CTα-deficient hepatocytes secreted larger HDL-sized particles than control hepatocytes (Fig. 2D). In plasma, however, HDL particles were slightly smaller in knock-out mice. The reason for the difference is currently unknown.

In summary, we show for the first time that hepatic PC biosynthesis is an important regulator of plasma HDL, and it contributes to the ongoing discussion regarding the role of the liver in HDL formation.

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