Hepatic Choline Transport Is Inhibited During Fatty Acid–Induced Lipotoxicity and Obesity

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Choline is an essential nutrient and a critical component of the membrane phospholipid phosphatidylcholine (PC), the neurotransmitter acetylcholine, while also contributing to the methylation pathway. In the liver specifically, PC is the major membrane constituent and can be synthesized by the cytidine diphosphate–choline or the phosphatidylethanolamine N-methyltransferase pathway. With the continuing global rise in the rates of obesity and nonalcoholic fatty liver disease, we sought to explore how excess fatty acids on primary hepatocytes and diet-induced obesity affect choline uptake and metabolism. Our results demonstrate that hepatocytes chronically treated with palmitate, but not oleate or a mixture, had decreased choline uptake, which was associated with lower choline incorporation into PC and lower expression of choline transport proteins. Interestingly, a reduction in the rate of degradation spared PC levels in response to palmitate when compared with control. The effects of palmitate treatment were independent of endoplasmic reticulum stress, which counterintuitively augmented choline transport and transporter expression. In a model of obesity-induced hepatic steatosis, male mice fed a 60% high-fat diet for 10 weeks had significantly diminished hepatic choline uptake compared with lean mice fed a control diet. Although the transcript and protein expression of various choline metabolic enzymes fluctuated slightly, we observed reduced protein expression of choline transporter-like 1 (CTL1) in the liver of mice fed a high-fat diet. Polysome profile analyses revealed that in livers of obese mice, the CTL1 transcript, despite being more abundant, was translated to a lesser extent compared with lean controls. Finally, human liver cells demonstrated a similar response to palmitate treatment. *Conclusion*: Our results suggest that the altered fatty acid milieu seen in obesity-induced fatty liver disease progression may adversely affect choline metabolism, potentially through CTL1, but that compensatory mechanisms work to maintain phospholipid homeostasis. (*Hepatology Communications* 2020;4:876-889).

In 1862, the German chemist Adolph Strecker isolated a strongly alkaline nitrogen-containing base and named it choline, which linked an earlier discovery by Theodore Gobley of Lécithine, now known to have been phosphatidylcholine (PC).[1] Choline is recognized as an essential nutrient that has critical functions as the precursor to the membrane phospholipid, PC, the precursor for the neurotransmitter, acetylcholine, as well as a methyl group donor.[2] In the liver, PC is the major membrane phospholipid and is primarily synthesized by the choline, cytidine diphosphate (CDP)–choline (Kennedy)

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CDP-choline, cytidine diphosphate–choline; CHKα, choline kinase alpha; ER, endoplasmic reticulum; FA, fatty acid; HFD, high-fat diet; KRH, Krebs-Ringer-HEPES; mRNA, messenger RNA; OCT, organic cation transporter; PC, phosphatidylcholine; Pcyt1a, phosphocholine cytidylyltransferase (protein also designated CCTα); PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine-N methyltransferase; siRNA, small interfering RNA; Slc44a1/2, solute carrier 44a1/2 (proteins also designated as choline transporter-like protein 1/2).

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pathway and supplemented with the methylation of phosphatidylethanolamine (PE) through the PE N-methyltransferase (PEMT) pathway.[3]

In the 1930s, Best and Huntsman first documented that choline deficiency was the root cause for the development of fatty liver in animals placed on an insufficient diet[4,5]; results that were later corroborated in humans.[6] Moreover, the importance of PC availability has become apparent using genetic mouse models. Targeted deletion of the first intracellular step of the CDP-choline pathway, choline kinase alpha (CHKα), and the rate-limiting step, phosphocholine cytidylyltransferase α (CCTα, encoded by the phosphocholine cytidylyltransferase [*Pcyt1a*] gene), are lethal *in utero*.[7,8] Liver-specific *Pcyt1a*-deficient mice had lower PC synthesis and higher liver triacylglycerol, in spite of a 2-fold increase in PEMT activity.[9] Finally, although *Pemt*-deficient mice fed a normal diet have minimal disruptions to hepatic phospholipid metabolism, when fed a choline-deficient diet, the lack of CDP-choline pathway flux results in complete liver failure within 3 days.[10]

Choline is a positively charged quaternary amine and requires transport through cellular membranes. Although the choline transporter SLC5A7 is a neuronal-specific, high-affinity choline transporter, in nonneuronal tissue, low-affinity and intermediate-affinity transporters have been described.[11] Organic cation transporters (OCT1-3, encoded by *Slc22a1-3* genes) uptake a broad range of organic cation substrates, xenobiotic compounds, various pharmaceutical drugs, and toxins.[12] However, given its affinity for choline (~200 μM), it remains unlikely that organic cation transporters represent a major transport system. To that end, almost 20 years ago, complementation experiments in yeast made possible the discovery of the choline transporter-like protein family (CTL1-5, encoded by the solute carrier family 44 members 1-5 [*Slc44a1-5*]).[13] Since then, CTL1 has been the most thoroughly studied member of this family and is thought to play a vital role in the uptake of choline along the plasma membrane as well as the outer mitochondrial membrane.[14]

The CDP-choline and PEMT pathways have been thoroughly investigated. Although early studies focused on hepatic choline uptake,[15,16] how this process is regulated under normal and pathological conditions remains unclear. Here we show that in mouse primary hepatocytes, choline transport is facilitated primarily by an intermediate-affinity transport system, most likely CTL1. To mimic hepatocyte lipotoxicity observed during metabolic dysfunction, we report that the saturated fatty acid (FA) palmitate, but not oleate or a combination, diminished choline uptake and PC synthesis, which was associated with lower CTL1 expression and independent of endoplasmic reticulum (ER) stress. Finally, in a mouse model of diet-induced obesity, hepatic choline uptake was diminished in obese livers, compared with lean controls. This was associated with a reduction in CTL1

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messenger RNA (mRNA) translation and protein levels. Given that choline deficiency is intimately linked to the instigation and potentially the progression of fatty liver, endogenous regulation of choline uptake may serve as an important and regulated process.

Experimental Procedures

ANIMALS

C57Bl/6J mice (stock no. 00064) were originally purchased from Jackson Laboratory (Bar Harbor, ME) and maintained as a breeding colony at the University of Ottawa (Ottawa, ON, Canada). Mice were exposed to a 12-hour light/dark cycle (7:00 AM/7:00 PM) and were fed *ad libitum* rodent diet (Envigo Teklad, Indianapolis, IN). Both male and female mice between 8 and 14 weeks were used for hepatocyte isolation. For diet-induced obesity studies, male C57Bl/6J mice were placed on a high-fat diet (HFD) (60% Kcal from fat; D12492) or matched control diet (10% Kcal from fat; D12450B) starting at 8 weeks of age for a duration of 10 weeks. Weight measurements were taken weekly. All of the experiments were performed with approval from the Animal Care Committee at the University of Ottawa.

STATISTICAL ANALYSES

All statistical analyses were completed using GraphPad Prism 7.03 (GraphPad Software Inc., San Diego, CA). Choline uptake saturation kinetic curves were generated using a nonlinear regression, Michaelis-Menten curve fit, whereas choline uptake inhibition curves were generated using a logarithmic (inhibitor) versus response curve fit. Comparisons between two groups were made using a paired Student *t* test, whereas comparisons between more than two treatment groups were made using a one-way analysis of variance (ANOVA) with a Tukey test for multiple comparisons, in which significant differences relative to bovine serum albumin (BSA) or vehicle control are shown. All data are expressed as mean ± SEM, unless specified in the figure legend. Further details regarding experimental procedures can be found in the Supporting Information.

Results

CHOLINE TRANSPORT IN PRIMARY HEPATOCYTES

Although hepatic PC metabolism has been studied extensively *in vitro* and *in vivo*, there have been no studies addressing the first step of the CDP-choline pathway, choline transport, specifically in primary hepatocytes. Therefore, we first sought to define the characteristics of choline transport in this culture system. In nonneuronal tissues, low-affinity (K_m > 200 μM) and intermediate-affinity (K_m ~ 50 μM) choline transport have been described.[11] In primary hepatocytes, choline uptake analyses revealed an intermediate affinity transport system (K_m = 61.36 ± 9.63 μM and V_{max} = 3.72 ± 0.34 nmol/mg/min) (Fig. 1A). Moreover, this choline transport activity was sensitive to the high-affinity and intermediate-affinity choline uptake inhibitor HC3 (Fig. 1B) and unresponsive to specific inhibition of OCTs by quinine (Fig. 1C), which has been shown effective at this dose in other cell culture systems.

EXOGENOUS FA TREATMENT ALTERS CHOLINE UPTAKE

The liver is a dynamic metabolic organ that under normal, healthy conditions, functions to balance lipid synthesis, uptake, and efflux. However, metabolic dysfunction associated with obesity and hepatic steatosis can result in a state of hepatocyte lipotoxicity.[17] To recapitulate this *in vitro*, we chronically (48 hours) treated hepatocytes with a mixture of palmitate and oleate (2:3, 0.5 mM) that has been thought to be representative of the most abundant FAs exposed to the liver *in vivo*, as well as palmitate and oleate in isolation, both at 0.5 mM. Following incubation, we observed a slight decrease in cell viability with FA treatments, which was more pronounced in the palmitate-containing treatments (Supporting Fig. S1). In response to FA treatment, the rate of choline uptake was diminished after FA treatment, with palmitate significantly inhibiting the rate of choline uptake in comparison with BSA-vehicle treated cells (Fig. 2).

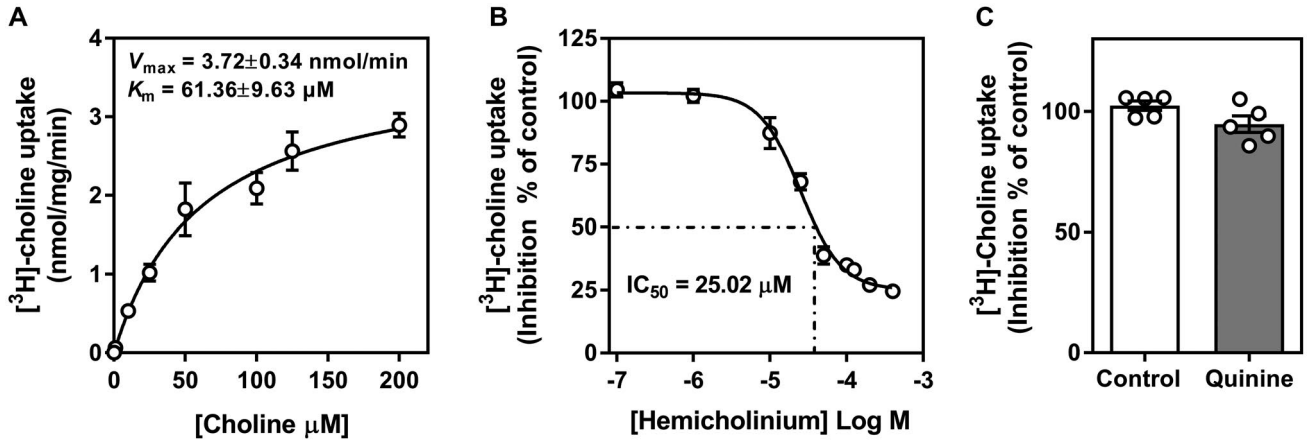

FIG. 1. Hepatocyte choline uptake occurs through intermediate affinity transport. (A) [^{3}H]-choline uptake in Krebs-Ringer-HEPES (KRH) buffer over 10 minutes in the presence of increasing amounts of unlabeled choline. (B) [^{3}H]-choline uptake in KRH buffer over 10 minutes in the presence of increasing amounts of choline uptake inhibitor HC3. (C) [^{3}H]-choline uptake in KRH buffer over 10 minutes in the presence of quinine, an organic cation transport inhibitor (100 μM). Data are expressed as the mean ± SEM from five separate isolations, each performed in triplicate.

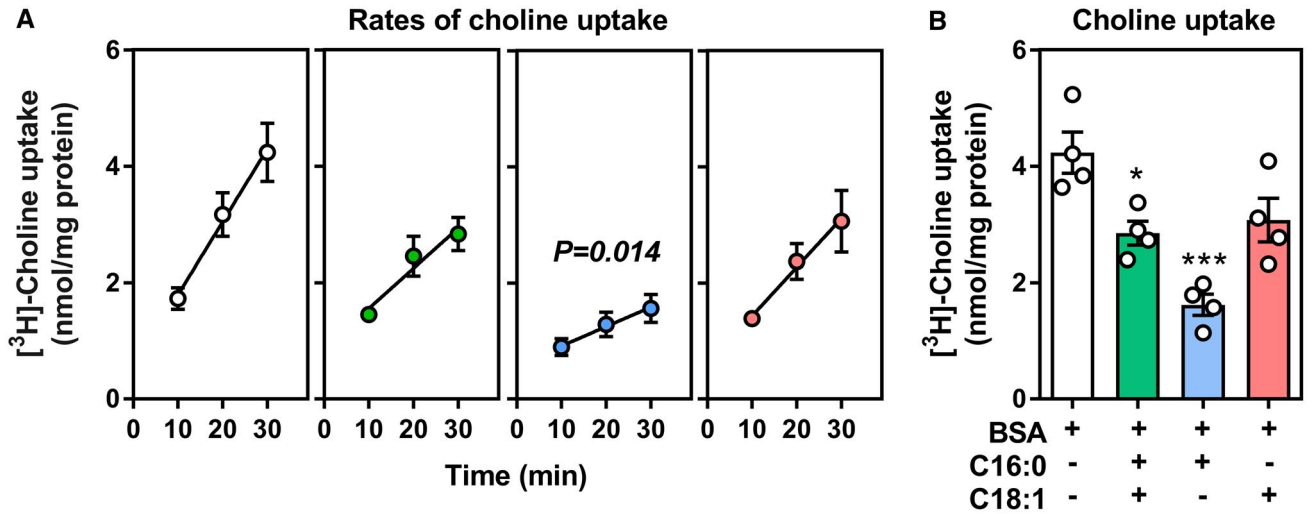

FIG. 2. Choline uptake is decreased with FA treatment. Hepatocytes were treated with a BSA/ethanol control, a 2:3 mixture of palmitate and oleate (0.5 mM), palmitate (0.5 mM), or oleate (0.5 mM) for 48 hours. (A) Following 1 hour in choline-free KRH buffer, the rate of [^{3}H]-choline uptake was assessed over the course of 30 minutes. (B) Intracellular [^{3}H]-choline at the 30-minute time point. Data are expressed as the mean ± SEM from four separate isolations, each performed in triplicate, in which the specific *P* value was determined by a comparison between the slopes derived through linear regression for BSA vehicle control and palmitate treatments. Statistical significance is shown as $^*P < 0.05$, $^{***}P < 0.001$ and $^{****}P < 0.0001$, compared with BSA vehicle control, and as determined by a one-way ANOVA with a Tukey test for multiple comparisons.

EXOGENOUS FA TREATMENT ALTERS CHOLINE UPTAKE

Because hepatocyte choline uptake was shown to be facilitated primarily by intermediate-affinity (CTL-mediated) transport, we next looked to measure the transcript and protein expression of the main choline transporters in hepatocytes. In response to both palmitate and oleate, but not the combination mixture, *Slc44a1* (which encodes CTL1) transcript expression was significantly lower compared with BSA control–treated cells (Fig. 3A). This trend was also observed at

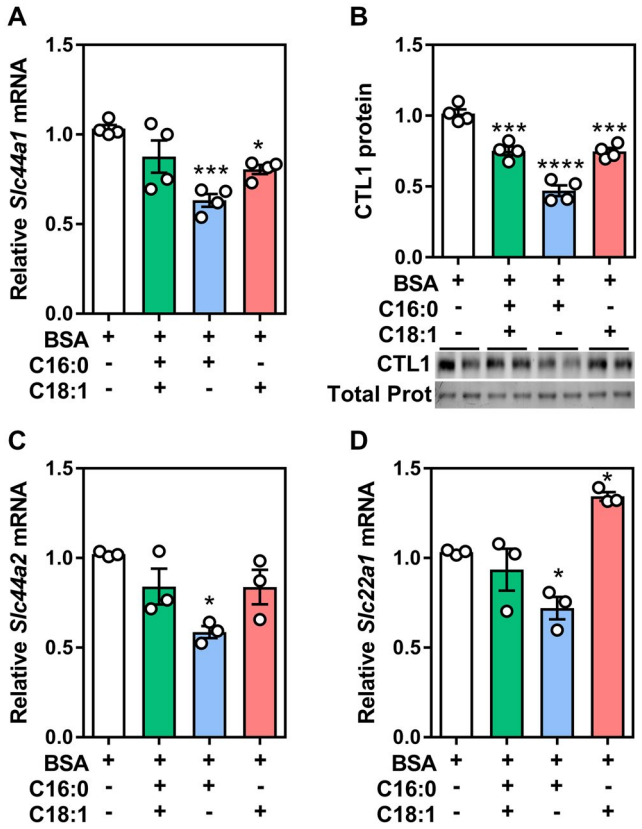

FIG. 3. Palmitate lowers choline transporter expression. Hepatocytes were treated with a BSA/ethanol control, 2:3 mixture of palmitate and oleate (0.5 mM), palmitate (0.5 mM), or oleate (0.5 mM) for 48 hours. (A) Relative mRNA expression of *Slc44a1*. (B) CTL1 protein content. (C) Relative mRNA expression of *Slc44a2*. (D) Relative mRNA expression of *Slc22a1*. mRNA expression was normalized to the average of *β-actin* and *Rplp0*. Data are expressed as the mean ± SEM from 3-4 separate isolations, each performed in triplicate. Statistical significance is shown as $^{*}P < 0.05$, $^{***}P < 0.001$ and $^{****}P < 0.0001$, compared with BSA vehicle control and as determined by a one-way ANOVA with a Tukey test for multiple comparisons.

the protein level, where after all FA treatments, CTL1 protein content was significantly diminished (Fig. 3B), which aligned well with the lower rate of choline uptake observed in FA-treated cells (Fig. 2). Although CTL1 has been implicated as the main choline transporter, CTL2 was also expressed in the liver and was significantly down-regulated at the transcript level in response to palmitate (Fig. 3C), but was undetectable at the protein level (data not shown). Interestingly, a divergent response was seen in the expression of the *Slc22a1* transcript, where palmitate lowered, but oleate increased its expression (Fig. 3D).

Choline uptake is the initial step in the CDP-choline pathway, where once internalized, choline destined for phospholipid synthesis is thought to be immediately phosphorylated by CHKα, shuttled to CCTα, and finally incorporated into PC by choline/ethanolamine phosphotransferase (CEPT). Although various FAs reduced choline uptake and the expression of CTL1 protein, only palmitate treatment down-regulated the expressions of *Chkα* and *Cept*, with no transcriptional changes in *Pcyt1a* (gene encoding CCTα) (Supporting Fig. S2A-C), similar to the regulatory gene in the CDP-ethanolamine pathway, phosphoethanolamine cytidylyltransferase (*Pcyt2*) (Supporting Fig. S2D). In addition to the CDP-choline pathway, the liver can convert PE to PC through the PE methylation pathway. Although it is known that PEMT activity in primary hepatocytes diminishes greatly after culturing,[18] we observed a slight, but statistically significant increase in the expression of *Pemt* with palmitate treatment (Supporting Fig. S2E). Therefore, while chronic palmitate/oleate-treated or oleate-treated hepatocytes experience a decrease in CTL1 protein content, palmitate treatment had a more global effect on the expression of choline transport, CDP-choline pathway, and PE methylation genes.

PALMITATE TREATMENT LOWERS CDP-CHOLINE PC SYNTHESIS AND DEGRADATION

To investigate the significance of altered choline uptake, we measured the incorporation of [^{3}H]-choline into PC after the 48-hour FA treatment. In a 4-hour pulse experiment, we observed a significant reduction in labeled choline incorporation into PC (Fig. 4A), entirely consistent with the reduced choline uptake (Fig. 2). To assess the total pool of PC, we monitored the incorporation of [^{3}H]-choline into PC over the duration of the 48-hour FA treatment, after which the labeled choline is anticipated to reach an equilibrium and represent steady-state levels of PC.[19,20] Interestingly, we saw no difference between any of the FA treatments compared with BSA control–treated cells (Fig. 4B). Given the apparent difference in PC synthesis through the CDP-choline pathway and the constant levels of PC, we next investigated the rate of PC degradation through pulse chase. After chronic FA treatment, palmitate/oleate or oleate had no effect. However, palmitate treatment significantly impaired

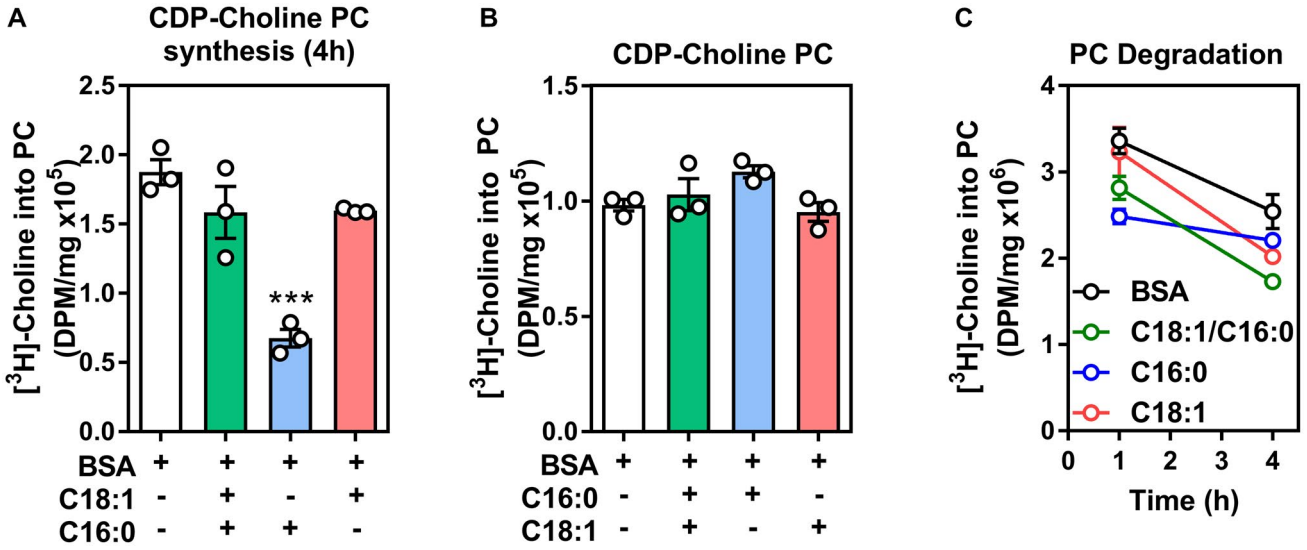

FIG. 4. PC metabolism, but not total content, is altered following FA treatment. Hepatocytes were treated with a BSA/ethanol control, 2:3 mixture of palmitate and oleate (0.5 mM), palmitate (0.5 mM), or oleate (0.5 mM) for 48 hours. The incorporation of [^{3}H]-choline into PC was measured 4 hours after FA treatment (A) or concurrently with the 48-hour treatment (B) as an indication of the total pool size of PC derived from the CDP-choline pathway. (C) The rate of PC degradation was assessed following FA treatment and following a 3-hour pulse with [^{3}H]-choline and 1-4 hour chase in the presence of excess unlabeled choline. Data are expressed as the mean ± SEM from three separate isolations, each performed in triplicate. Statistical significance is shown as $^{***}P < 0.001$, compared with BSA vehicle control, and as determined by one-way ANOVA with a Tukey test for multiple comparisons. Abbreviation: DPM, disintegrations per minute.

the rate at which PC was degraded, likely contributing to its maintained levels (Fig. 4C).

PALMITATE TREATMENT DOES NOT ALTER THE CDP-ETHANOLAMINE OR PEMT PATHWAY

Given that reductions in choline uptake observed with FA (primarily palmitate) treatment altered PC synthesis, we next assessed whether chronic lipid loading of hepatocytes altered the incorporation of ethanolamine into PE. Although there was no change between conditions in the short-term incorporation of labeled ethanolamine into PE (Fig. 5A), palmitate/oleate and palmitate reduced total levels of PE, compared with BSA control–treated cells (Fig. 5B). Finally, to test the role of PE methylation under these conditions, we attempted to measure the amount of [^{14}C]-ethanolamine incorporated into PE and then converted to PC during a 4-hour labeling period following the FA treatment. However, we were unable to measure any radioactivity in PC after [^{14}C]-ethanolamine pulse (data not shown), which is consistent with previous reports that PEMT activity dramatically decreases after hepatocytes are cultured for more than 24 hours.[18] To have an indication as to the partial contribution of the PEMT pathway in these cells, we performed chronic labeling experiments, in which cells were co-incubated with FAs as well as [^{14}C]-ethanolamine for 48 hours. Under these conditions, we observed no difference in PEMT-derived PC between any of the FA treatments, compared with BSA control–treated cells (Fig. 5C).

ER STRESS IS NOT RESPONSIBLE FOR FA-INDUCED REDUCTIONS IN CHOLINE UPTAKE

It is well documented that hepatic exposure to high and chronic levels of saturated FAs leads to ER stress, leading to metabolic dysfunction and induction of cell death pathways.[17] We reasoned that palmitate-induced reductions in choline uptake, CTL1, and choline incorporation into PC might be due to palmitate-induced ER stress. To test this, we used the

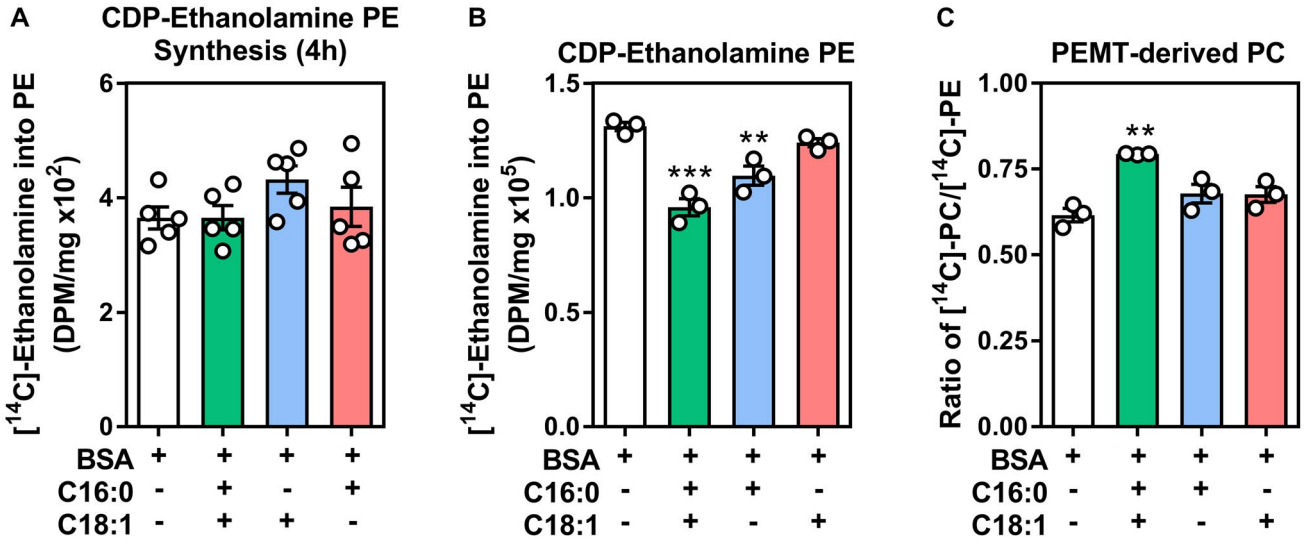

FIG. 5. PE metabolism but not PEMT pathway is altered following FA treatment. Hepatocytes were treated with a BSA/ethanol control, 2:3 mixture of palmitate and oleate (0.5 mM), palmitate (0.5 mM), or oleate (0.5 mM) for 48 hours. The incorporation of [^{14}C]-ethanolamine into PE was measured 4 hours after FA treatment (A) or concurrently with the 48-hour treatment (B) as an indication of the total pool size of PE derived from the CDP-ethanolamine pathway. (C) PC derived from the methylation of PE through the PEMT pathway is displayed as the percentage of PC/PE generated through [^{14}C]-ethanolamine labeling. Data are expressed as the mean ± SEM from three separate isolations, each performed in triplicate. Statistical significance is shown as $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with BSA vehicle control, and as determined by a one-way ANOVA with a Tukey test for multiple comparisons.

well-known inducer of ER stress, tunicamycin,[21] which only slightly reduced cell viability at the higher dose (Supporting Fig. S3). Contrary to our hypothesis, although FA treatment inhibited choline uptake, induction of ER stress resulted in a dose-dependent increase in choline transport (Fig. 6A). This was accompanied by significant increases in choline transporter (*Slc44a1*, *Slc44a2*, and *Slc22a1*) transcript expressions (Fig. 6B-D). However, despite validation of ER stress using known markers, CTL1 and CTL2 protein content were not changed compared with vehicle control (Fig. 6E).

ER STRESS LEADS TO MARKED REDUCTION IN CDP-CHOLINE PC FLUX

Since we observed that tunicamycin-induced ER stress augmented choline uptake, we anticipated that the incorporation of choline into PC would follow suit. However, we saw a dramatic reduction in the 4-hour incorporation of labeled choline into PC (Fig. 7A). When we incubated tunicamycin-treated cells concurrently with [^{3}H]-choline or [^{14}C]-ethanolamine for 48 hours to estimate total PC and PE, respectively, there was a dose-dependent decrease in the total levels of both phospholipids, as generated by the CDP-choline (Fig. 7B) and CDP-ethanolamine pathways (Fig. 7C), with no net change in PEMT-derived PC (Fig. 7D). Interestingly, the transcript expression of CDP-choline and CDP-ethanolamine pathway genes were increased at the lower dose of tunicamycin, compared with the vehicle control (Supporting Fig. S4A-D). There was no difference in the expression of *Pemt* (Supporting Fig. S4E).

CHOLINE UPTAKE IS LOWER IN RESPONSE TO PALMITATE AND CTL1-DEPENDENT IN HUMAN HEPATIC CELLS

Finally, we probed whether human hepatocyte–derived cells underwent similar changes in response to FA treatments. Choline uptake was dramatically reduced in HepG2 cells when treated with the same concentration of palmitate (0.5 mM), which was primarily attributed to poor viability (data not shown). Interestingly, oleate treatment strongly and significantly augmented choline transport (Supporting Fig. S5). When the concentration of palmitate was

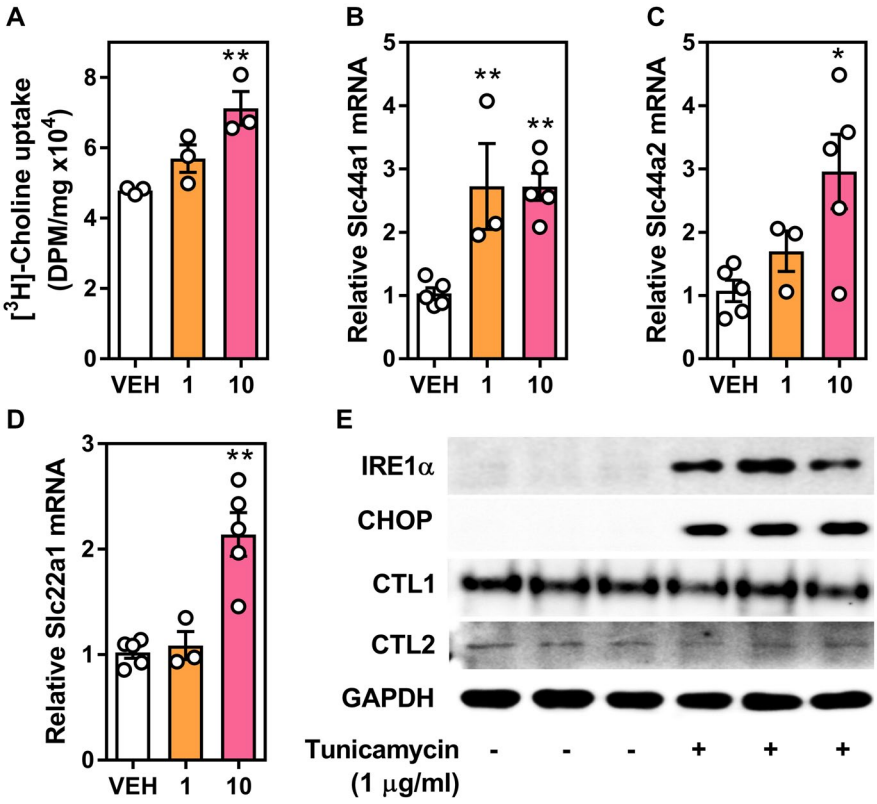

FIG. 6. Tunicamycin-induced ER stress augments choline uptake and choline transporter transcript expression. Hepatocytes were treated with tunicamycin (1 and 10 μg/mL) for 48 hours. Following treatment, [^{3}H]-choline uptake was measured (A) as well as the relative transcript expressions of *Slc44a1* (B), *Slc44a2* (C) and *Slc22a1* (D), all normalized to the expressions of *β-actin* and *Rplp0*. (E) Protein expression of IRE1α, CHOP, CTL1, and CTL2 following treatment with 1 ug/mL of tunicamycin. Data are expressed as the mean ± SEM from three to five separate isolations, each performed in triplicate. Statistical significance is shown as $^*P < 0.05$ and $^{**}P < 0.01$, compared to vehicle control, and as determined by a one-way ANOVA with a Tukey test for multiple comparisons. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; and VEH, vehicle.

lowered to a more tolerable level (0.2 mM), choline transport remained lower; however, unlike primary mouse hepatocytes and obese mouse livers, this was not seemingly related to transcript or protein expression of CTL1 or other choline transporters (Fig. 8). Interestingly, consistent with primary mouse hepatocytes, there were no differences in total PC levels, likely due to compensatory mechanisms to maintain proper phospholipid ratios (Supporting Fig. S5B). As a final measure to mechanistically link, we interrogated whether targeted disruption of CTL1 is causally linked to lower choline transport. In the human Huh7.5 hepatic cell line, use of an anti-CTL1 antibody or small interfering RNA (siRNA) knockdown lowered choline uptake to a similar level when compared to HC3 (100 μM), a broad-acting inhibitor of choline transport (Supporting Fig. S5C,D). These results provide evidence that support a role for CTL1 in mediating most of the hepatocyte choline transport.

LIVER CHOLINE UPTAKE IS REDUCED DURING OBESITY-INDUCED HEPATIC STEATOSIS

We next sought to validate the *in vivo* relevance of exogenous FA treatment of isolated primary hepatocytes. Male C57Bl/6J mice were either fed an obesogenic high-fat (60% Kcal from fat) or a control (10% Kcal from fat) diet starting at 8 weeks of age for 10 weeks. As expected, mice fed the HFD gained significantly more weight compared with control-fed mice (Fig. 9A). We next injected mice with a dose of [^{3}H]-choline to track the short-term uptake and incorporation of choline into PC in the liver and

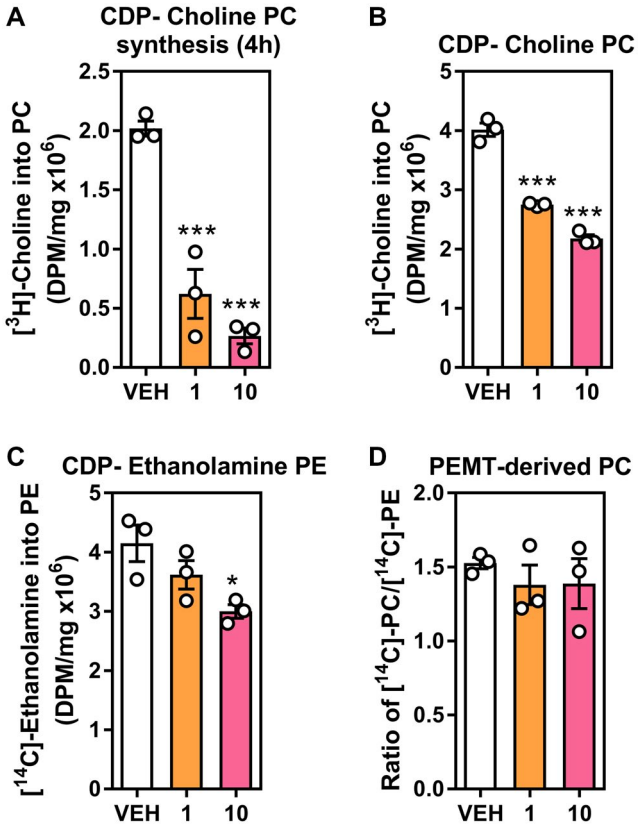

FIG. 7. ER stress down-regulates phospholipid synthesis. Hepatocytes were treated with tunicamycin (1 and 10 μg/mL) for 48 hours. The incorporation of [^{3}H]-choline into PC was measured 4 hours after (A) or concurrently with the 48-hours tunicamycin treatment (B) as an indication of the total pool size of PC derived from the CDP-choline pathway. (C) Total pool size of PE derived from the CDP-ethanolamine pathway. (D) PC derived from PEMT following incorporation of [^{14}C]-ethanolamine into lipids during the 48-hour concurrent treatment, in which PC derived from the methylation of PE is displayed as the percentage of PC/PE generated through [^{14}C]-ethanolamine labeling. Data are expressed as the mean ± SEM from three separate isolations, each performed in triplicate. Statistical significance is shown as $^*P < 0.05$ and $^{***}P < 0.001$, compared with vehicle control, and as determined by a one-way ANOVA with a Tukey test for multiple comparisons. Abbreviation: VEH, vehicle.

observed that when normalized for circulating choline and total protein content, obese livers had significantly less choline uptake and incorporation into PC (Fig. 9B and Supporting Fig. S6). This was in keeping with a total reduction in the steady-state level of PC in the livers of obese mice that were previously documented.[22-24]

Interestingly, when we assessed the transcript levels of choline transporters, *Slc44a1* was higher (Fig. 10A), there was no change in *Slc44a2*, and *Slc22a1* was lower (Supporting Fig. S6A,B). However, at the protein level, CTL1 in obese livers was significantly lower compared with lean controls, whereas there was no difference in CTL2 or OCT1 protein content (Fig. 10B and Supporting Fig. S6C). There were also no differences in transcript or protein content of CHKα and CCTα (Supporting Fig. S6C-F). Given the discordance between CTL1 transcript and protein expression in lean and obese livers, we next sought to determine whether CTL1 mRNA was translationally repressed. We performed polysome fractionation on lean and obese livers, and the mRNA distribution of *Slc44a1* and a housekeeping control (*Hprt*, the gene encoding hypoxanthine-guanine phosphoribosyltransferase) were assessed across the fractions representing free mRNA (fractions 7-10), monosome (fractions 11-12), light polysome (fractions 13-15), and heavy polysome (fractions 16-19). In line with a diminished protein content, in the livers of obese mice, we observed that *Slc44a1*, but not *Hprt*, was shifted toward the monosome fractions and away from the heavy polysome fractions, as compared with lean controls (Fig. 10C and Supporting Fig. S6G).

Discussion

Choline is an essential nutrient that needs to be obtained from dietary sources to supplement low levels of endogenous synthesis. The liver is one of the main sites of choline metabolism, as it has the capacity for both PC synthesis as well as mitochondrial oxidation, the first and irreversible step toward its incorporation as a methyl donor. Through its integration into PC, hepatic choline availability governs many aspects of hepatic phospholipid metabolism, such as triglyceride synthesis and very low-density formation.[3,25] Although there has been a concerted effort to map the fate of hepatic choline once inside the cell, there have been few reports that have aimed to characterize the specific transport of choline under normal or pathological conditions.

Here, we report that in primary murine hepatocytes, choline is transported through an intermediate affinity system that is consistent with the choline transporter-like family of proteins, of which CTL1 and CTL2 represent the most likely candidates. Although there have been other reports that have characterized choline transporters in various immortalized cell lines,[11,26]

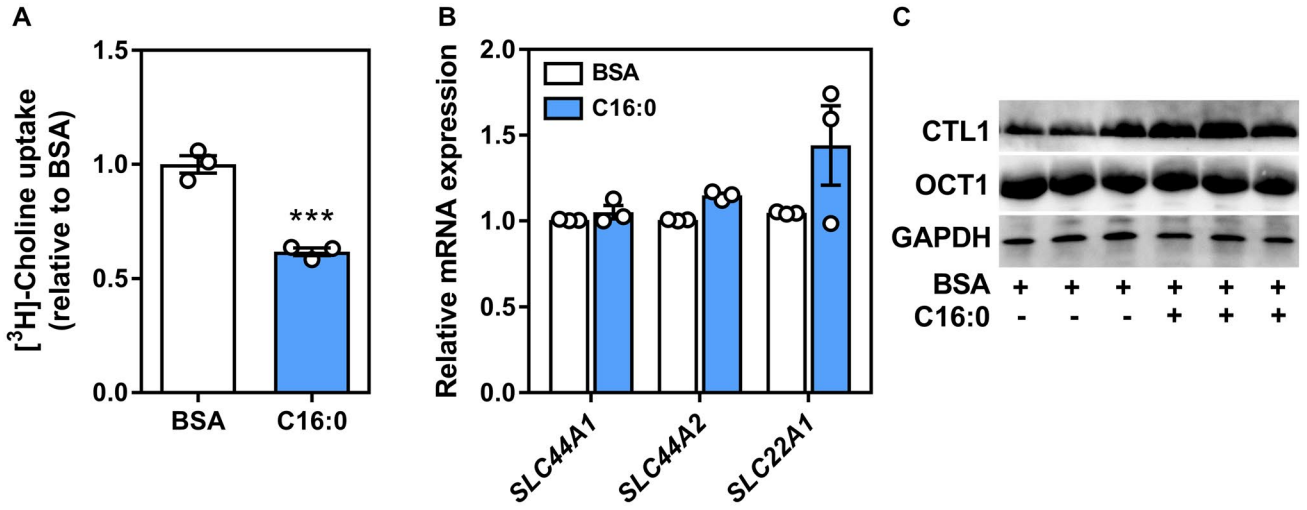

FIG. 8. Choline uptake is decreased with FA treatment in human HepG2 cells. Cells were seeded at 30% confluence and treated with a BSA/ethanol control or palmitate (0.2 mM) for 48 hours. (A) Following 1 hour in choline-free KRH buffer, the rate of [^{3}H]-choline uptake was assessed over the course of 30 minutes. (B) Relative mRNA expression of *SLC44A1*, *SLC44A2,* and *SLC22A1*. (C) Relative mRNA expression of CTL1 and OCT1 protein content (CTL2 protein was no detected). mRNA expression was normalized to the average of *β-ACTIN* and *RPLP0*. Data are expressed as the mean ± SEM (n = 3), each performed in triplicate. Statistical significance is shown as $^{***}P < 0.001$, compared with BSA vehicle control, as determined by a Student *t* test. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

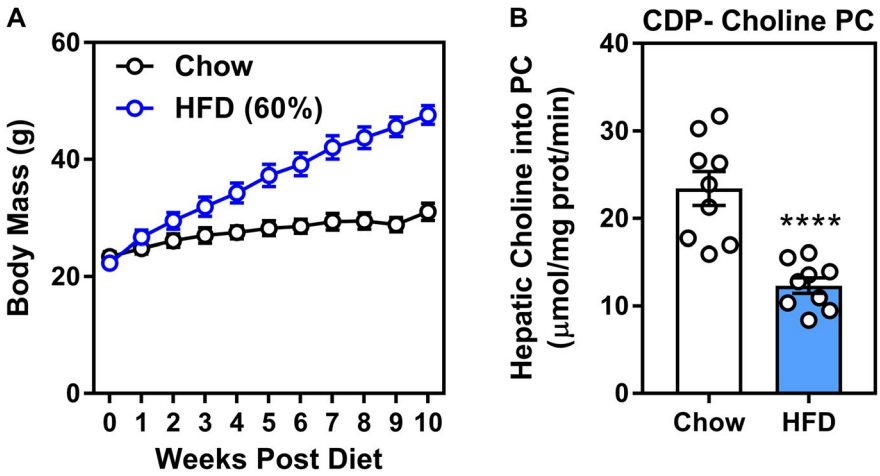

FIG. 9. Choline uptake is lower in obese livers compared with lean control. Male C57Bl/6J mice were fed a 60% high-fat or 10% control (Chow) diet for 10 weeks. (A) Weekly body weights. (B) [^{3}H]-choline was injected into mice, and the uptake and incorporation into PC in the liver was determined after 1 hour. Data are expressed as the mean ± SEM (n = 9 per dietary group). Statistical significance is shown as $^{****}P < 0.0001$, compared with vehicle lean control mice, as determined by a Student *t* test.

many of which are cancer-derived, and very little focus has been given to understanding choline transport in primary cells. Moreover, although we demonstrate evidence for the CTL family as being the main facilitators of choline uptake, the relative contribution of each protein remains unknown. Past studies have used siRNA and antibody occlusion to tease out a primary role for CTL1 in macrophage biology[27,28]; however, until now such an approach had not been mirrored in hepatocytes. Here we show that in human hepatocyte–derived Huh7.5 cells, antibody blockage and siRNA-mediated knockdown of CTL1 is on par with

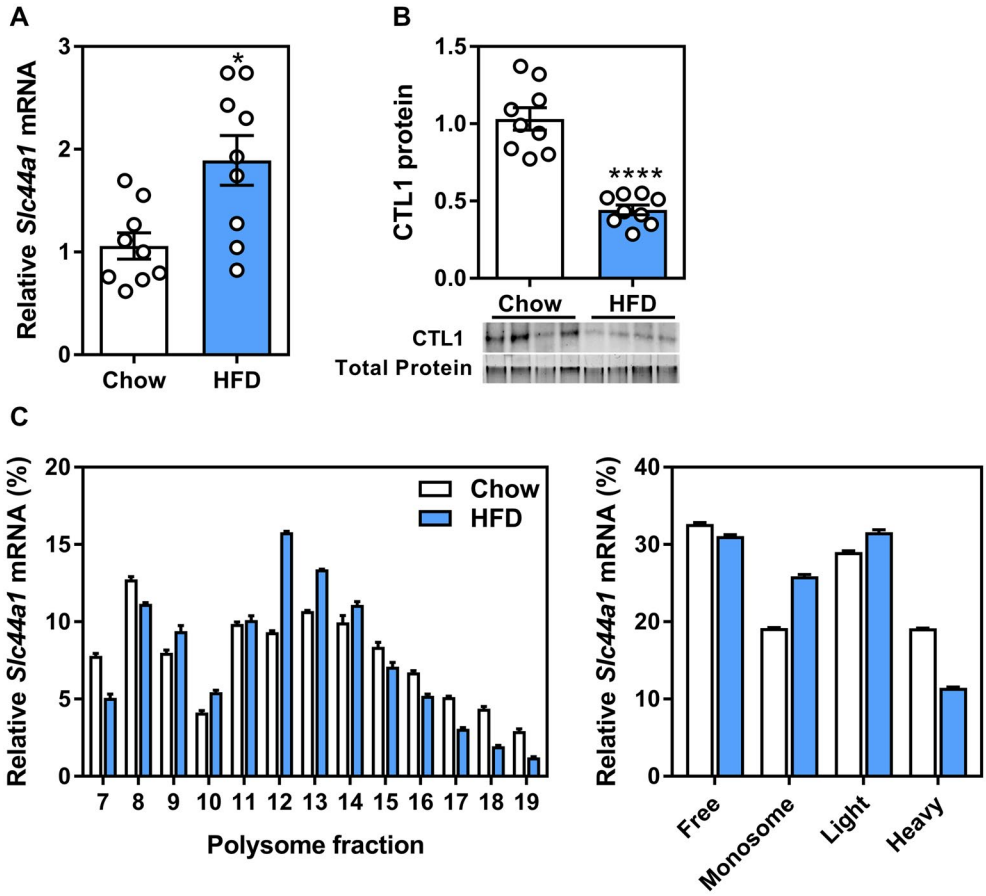

FIG. 10. Obesity is associated with lower translation of CTL1. *Slc44a1* transcript (A) and CTL1 (B) total protein content was assessed in livers from lean and obese mice. mRNA expression was normalized to the average of *β-actin* and *Rplp0*, in which protein expression was compared with the stain of total protein. Data are expressed as the mean ± SEM (n = 9 per dietary group). Statistical significance is shown as $^{****}P < 0.0001$, compared with vehicle lean control mice, as determined by a Student *t* test. (C) Polysome fractions were isolated and the expression of *Slc44a1* was assessed in each (left). Relative expression was also displayed in the fractions representing free transcript (7-10), monosomes (11-12), lightly translated (13-15), and heavily translated (16-19) (right). Data are expressed as the mean ± SEM and are representative of two independent experiments completed with technical triplicates, shown relative to *β-actin*.

the pan-choline uptake inhibitor HC3. This strongly suggests that CTL1 is the main choline transporter in hepatic cells and that choline transport is highly linked to both its expression and function.

It is well known that limiting choline availability or hepatic choline metabolism drives patterns of altered lipid homeostasis.[9,29-32] Early studies by Charles Best and others documented that liver dysfunction associated with choline deficiency was reversed by choline supplementation through delivery of PC.[4,5] Moreover, while considered a dramatic model, choline and methionine deficiency very rapidly induces hepatic steatosis that has some of the key hallmarks of human nonalcoholic fatty liver disease.[33] However, given the continuing rise in the rates of obesity and fatty liver disease, we considered that choline uptake as an initiator of choline incorporation into PC through the CDP-choline pathway might be affected by the onset of hepatic lipid accumulation. To model this *in vitro*, we isolated primary hepatocytes and treated cells with a mixture or individual FAs that are well documented to both mirror circulating FA profiles during obesity[34] as well as mimic the increased lipid burden in hepatocytes.[35] While FA treatment induced a certain level of cellular stress as expected, cell viability was well within the expected range, given saturated fat treatment (Supporting Fig. S1). Previous reports have documented a more dramatic effect on markers of cell death and viability with palmitate treatment,

at or above the amount used in our study (0.5 mM) and for less duration.[36,37] In our study, it does not appear as though the FA-induced reductions in choline uptake are due to diminished cell viability. It should be mentioned that in human HepG2 cells, a lower concentration of palmitate (0.2 mM) was necessary due to viability issues.

While our results demonstrate that in hepatocytes chronically treated (48 hours) with FAs, choline uptake tended to decrease—independent of the type of lipid; palmitate alone had the clearest inhibitory effect. This was associated with a reduction in total protein content of CTL1 that can likely be explained by a similar decrease in transcript levels. It is interesting to note that the levels of *Slc44a1* are not significantly diminished after only 24 hours of FA treatment (data not shown), which suggests that the transcriptional repression happens on a more chronic scale. The expression of other choline transporters and CDP-choline pathway genes was also lower with palmitate; however, protein levels were not assessed. Moreover, independent of these down-regulated genes and lower CTL1 protein content, measures of total PC were no different among any of the treatments. We observed that the degradation of PC was significantly reduced in palmitate-treated cells compared with control, which is a surrogate measure for phospholipase D activity. We reasoned it possible that reductions in choline uptake were facilitated by the reduced expression of CTL1, but that degradation was diminished to account for the lower amount of PC produced, similar to adaptations that occur when the CDP-ethanolamine pathway is diminished.[38] However, it remains possible that (1) PC synthesis is normal, and differences in choline transport mask this effect with radiolabeling; and 2) similar to choline deficiency in hepatocytes, the availability of phosphocholine (the product of CHKα) is sufficiently above the K_m of CCTα, such that the levels of choline converted to phosphocholine were never limiting.[39] Moreover, future work involving specific knockdown and/or overexpression of *Slc44a1*/CTL1 will be necessary to ascribe causality to its up-regulation or down-regulation. Interestingly, very recent findings have characterized rare homozygous loss-of-function mutations in the *SLC44A1* gene in humans, presenting as childhood-onset neurodegeneration.[40]

Previous links have been made between FA-induced changes and choline metabolism. In the immortalized mouse myoblast C2C12 cell line, palmitate was shown to limit cellular choline uptake, which was associated with increased lysosomal degradation of CTL1, whereas oleate was seen to decrease not only mitochondrial choline uptake, but stimulate PC synthesis overall.[41] In our hepatocyte experiments, we observed that palmitate, but not oleate, lowered *Slc44a1* transcript expression, which resulted in an overall reduction of CTL1 protein. We did not evaluate mitochondrial localization of CTL1, nor did we evaluate the fate of oxidized choline, which occurs in the mitochondria in liver cells. Therefore, it is possible that in hepatocytes, there are effects of saturated and monounsaturated FAs that are going unreported. Recently, in *Pcyt1a*-deficient macrophages, a decreased rate of PC turnover facilitated a shift toward more polyunsaturated FAs in membrane phospholipids, which stemmed their inflammatory potential.[42] Although our study focused on the consequence of exogenous FA treatment on choline transport and subsequent metabolism, we learned that (1) PC metabolism can be regulated because of pathway interruptions and (2) it is possible that alterations in phospholipid FA composition might alter the membrane properties that can affect transport proteins.[43] Finally, many studies have now linked phospholipid biosynthetic pathways with diacylglycerol and triglyceride synthesis, storage, and metabolism.[9,19,20,28,44] There remains the possibility that exogenous FAs might feedback to affect choline metabolism through neutral lipid homeostasis; however, this was not addressed here.

Given that palmitate-induced changes on choline metabolism were consistently the most apparent, we hypothesized that ER stress may be the root cause of this effect. However, when we used the well-known ER stress-inducing agent tunicamycin,[21] choline uptake, counter to our expectations, increased. This was associated with a consistent increase in the transcript expression of choline transporters with no change in the total levels of CTL1 or CTL2 protein. It remains entirely possible that rather than an increase in protein content, the distribution of CTL1 (or other transporters) shifted from intracellular to membrane, thus facilitating the increase in choline uptake, which may similarly explain lower choline transport in palmitate-treated HepG2 cells independent of transporter expression. Despite there being an increase in the acute uptake of choline, the incorporation of choline and ethanolamine into their respective phospholipids was greatly diminished and was not compensated by increases in

PEMT protein amounts. This was also independent of a general increase in the gene expression of many CDP-choline and CDP-ethanolamine pathways transcripts, although it might be that translation of these transcripts was generally suppressed given the level of ER dysfunction. Because ER homeostasis is critical for phospholipid biosynthetic enzymes, this was perhaps not surprising.[45] Therefore, although palmitate is well known for inducing ER stress, a separate and yet unknown mechanism is responsible for diminishing choline uptake in hepatocytes.

There have been no studies addressing the hepatic transport of choline in a model of obesity, although total PC content has been shown to be reduced.[22-24] In keeping with primary hepatocytes treated with saturated fat, we observed that obese livers had a reduced amount of choline taken into the cell and converted to PC, as compared with lean animals. We observed a concomitant decrease in CTL1 protein expression that was driven by *Slc44a1* mRNA translational repression. Although it has been shown that mechanistic target of rapamycin complex 1 is able to control hepatic PC levels through translational regulation of CCTα,[46] CTL1 was not examined. Moreover, while the observed reduction in both CTL1 protein content and functional choline transport *in vivo* remains correlative, understanding the specific translational control of choline and lipid metabolism genes and the importance of CTL1 to hepatic phospholipid metabolism during obesity remain relevant future questions. Interestingly, while evidence in humans also remains correlative, hepatic *Slc44a1* expression is down-regulated in the progression of fatty liver,[47] single nucleotide polymorphisms in *Slc44a1* predispose individuals to dysregulated metabolism,[48] and loss of function mutations of *Pcyt1a* causes severe hepatic dysfunction.[49]

In conclusion, we show in primary hepatocytes that choline is primarily taken into the cell through intermediate affinity transporters, and that in response to exogenous FAs, choline transport and PC synthesis was suppressed. However, total PC levels were maintained through a reduction in PC turnover. FA-induced disruption to choline transport was not mediated by the induction of ER stress. Finally, in diet-induced model of obesity, hepatic choline transport was diminished and associated with lower protein synthesis of CTL1. Our results suggest that the metabolism (uptake, synthesis, and degradation) of choline and PC are altered in pathological states of obesity, which are highly associated with the initiation of nonalcoholic fatty liver disease.

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REFERENCES

1) Zeisel SH. A brief history of choline. Ann Nutr Metab 2012;61:254-258.
2) Ueland PM. Choline and betaine in health and disease. J Inherit Metab Dis 2011;34:3-15.
3) van der Veen JN, Kennelly JP, Wan S, Vance JE, Vance DE, Jacobs RL. The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. Biochim Biophys Acta Biomembr 2017;1859:1558-1572.
4) Best CH, Huntsman ME. The effect of choline on the liver fat of rats in various states of nutrition. J Physiol 1935;83:255-274.
5) Best CH, Huntsman ME. The effects of the components of lecithine upon deposition of fat in the liver. J Physiol 1932;75: 405-412.
6) Zeisel SH, Da Costa KA, Franklin PD, Alexander EA, Lamont JT, Sheard NF, et al. Choline, an essential nutrient for humans. FASEB J 1991;5:2093-2098.
7) Wu G, Aoyama C, Young SG, Vance DE. Early embryonic lethality caused by disruption of the gene for choline kinase alpha, the first enzyme in phosphatidylcholine biosynthesis. J Biol Chem 2008;283:1456-1462.
8) Wang L, Magdaleno S, Tabas I, Jackowski S. Early embryonic lethality in mice with targeted deletion of the CTP:phosphocholine cytidylyltransferase alpha gene (Pcyt1a). Mol Cell Biol 2005;25: 3357-3363.
9) Jacobs RL, Devlin C, Tabas I, Vance DE. Targeted deletion of hepatic CTP:phosphocholine cytidylyltransferase alpha in mice decreases plasma high density and very low density lipoproteins. J Biol Chem 2004;279:47402-47410.
10) Walkey CJ, Yu L, Agellon LB, Vance DE. Biochemical and evolutionary significance of phospholipid methylation. J Biol Chem 1998;273:27043-27046.
11) Traiffort E, O'Regan S, Ruat M. The choline transporter-like family SLC44: properties and roles in human diseases. Mol Aspects Med 2013;34:646-654.
12) Koepsell H, Lips K, Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharm Res 2007;24:1227-1251.
13) O'Regan S, Traiffort E, Ruat M, Cha N, Compaore D, Meunier FM. An electric lobe suppressor for a yeast choline transport mutation belongs to a new family of transporter-like proteins. Proc Natl Acad Sci U S A 2000;97:1835-1840.
14) Michel V, Bakovic M. The solute carrier 44A1 is a mitochondrial protein and mediates choline transport. FASEB J 2009;23: 2749-2758.
15) Sinclair CJ, Chi KD, Subramanian V, Ward KL, Green RM. Functional expression of a high affinity mammalian hepatic choline/organic cation transporter. J Lipid Res 2000;41:1841-1848.
16) Zeisel SH, Story DL, Wurtman RJ, Brunengraber H. Uptake of free choline by isolated perfused rat liver. Proc Natl Acad Sci U S A 1980;77:4417-4419.
17) Peters KM, Wilson RB, Borradaile NM. Non-parenchymal hepatic cell lipotoxicity and the coordinated progression of nonalcoholic fatty liver disease and atherosclerosis. Curr Opin Lipidol 2018;29:417-422.

18) Vance DE. Phospholipid methylation in mammals: from biochemistry to physiological function. Biochim Biophys Acta 2014;18:1477-1487.
19) Fullerton MD, Hakimuddin F, Bonen A, Bakovic M. The development of a metabolic disease phenotype in CTP:phosphoethanolamine cytidylyltransferase-deficient mice. J Biol Chem 2009;284:25704-25713.
20) Fullerton MD, Bakovic M. Complementation of the metabolic defect in CTP:phosphoethanolamine cytidylyltransferase (Pcyt2)-deficient primary hepatocytes. Metabolism 2010;59:1691-1700.
21) Abdullahi A, Stanojcic M, Parousis A, Patsouris D, Jeschke MG. Modeling acute ER stress *in vivo* and *in vitro*. Shock 2017;47:506-513.
22) Eisinger K, Krautbauer S, Hebel T, Schmitz G, Aslanidis C, Liebisch G, et al. Lipidomic analysis of the liver from high-fat diet induced obese mice identifies changes in multiple lipid classes. Exp Mol Pathol 2014;97:37-43.
23) Kim HJ, Kim JH, Noh S, Hur HJ, Sung MJ, Hwang JT, et al. Metabolomic analysis of livers and serum from high-fat diet induced obese mice. J Proteome Res 2011;10:722-731.
24) Puri P, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O, et al. A lipidomic analysis of nonalcoholic fatty liver disease. Hepatology 2007;46:1081-1090.
25) van der Veen JN, Lingrell S, Vance DE. The membrane lipid phosphatidylcholine is an unexpected source of triacylglycerol in the liver. J Biol Chem 2012;287:23418-23426.
26) **Gobeil Odai K**, **O'Dwyer C**, **Steenbergen R**, Shaw TA, Renner TM, Ghorbani P, et al. *In vitro* hepatitis C virus infection and hepatic choline metabolism. Viruses 2020;12:E108
27) Sanchez-Lopez E, Zhong Z, Stubelius A, Sweeney SR, Booshehri LM, Antonucci L, et al. Choline uptake and metabolism modulate macrophage IL-1β and IL-18 production. Cell Metab 2019;29:1350-1362.e1357.
28) **Snider SA**, **Margison KD**, Ghorbani P, LeBlond ND, O'Dwyer C, Nunes JRC, et al. Choline transport links macrophage phospholipid metabolism and inflammation. J Biol Chem 2018;293:11600-11611.
29) Buchman AL, Dubin MD, Moukarzel AA, Jenden DJ, Roch M, Rice KM, et al. Choline deficiency: a cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. Hepatology 1995;22:1399-1403.
30) Buchman AL, Ament ME, Sohel M, Dubin M, Jenden DJ, Roch M, et al. Choline deficiency causes reversible hepatic abnormalities in patients receiving parenteral nutrition: proof of a human choline requirement: a placebo-controlled trial. JPEN J Parenter Enteral Nutr 2001;25:260-268.
31) Jacobs RL, Zhao Y, Koonen DP, Sletten T, Su B, Lingrell S, et al. Impaired de novo choline synthesis explains why phosphatidylethanolamine N-methyltransferase-deficient mice are protected from diet-induced obesity. J Biol Chem 2010;285:22403-22413.
32) Sanchez-Lopez E, Zimmerman T, Gomez del Pulgar T, Moyer MP, Lacal Sanjuan JC, Cebrian A. Choline kinase inhibition induces exacerbated endoplasmic reticulum stress and triggers apoptosis via CHOP in cancer cells. Cell Death Dis 2013;4:e933.
33) Mehedint MG, Zeisel SH. Choline's role in maintaining liver function: new evidence for epigenetic mechanisms. Curr Opin Clin Nutr Metab Care 2013;16:339-345.
34) Soriguer F, Garcia-Serrano S, Garcia-Almeida JM, Garrido-Sanchez L, Garcia-Arnes J, Tinahones FJ, et al. Changes in the serum composition of free-fatty acids during an intravenous glucose tolerance test. Obesity (Silver Spring) 2009;17:10-15.
35) de Almeida IT, Cortez-Pinto H, Fidalgo G, Rodrigues D, Camilo ME. Plasma total and free fatty acids composition in human non-alcoholic steatohepatitis. Clin Nutr 2002;21:219-223.
36) Shen C, Dou X, Ma Y, Ma W, Li S, Song Z. Nicotinamide protects hepatocytes against palmitate-induced lipotoxicity via SIRT1-dependent autophagy induction. Nutr Res 2017;40:40-47.
37) Jung TW, Hong HC, Hwang HJ, Yoo HJ, Baik SH, Choi KM. C1q/TNF-related protein 9 (CTRP9) attenuates hepatic steatosis via the autophagy-mediated inhibition of endoplasmic reticulum stress. Mol Cell Endocrinol 2015;417:131-140.
38) Fullerton MD, Hakimuddin F, Bakovic M. Developmental and metabolic effects of disruption of the mouse CTP: phosphoethanolamine cytidylyltransferase gene (Pcyt2). Mol Cell Biol 2007;27:3327-3336.
39) Kulinski A, Vance DE, Vance JE. A choline-deficient diet in mice inhibits neither the CDP-choline pathway for phosphatidylcholine synthesis in hepatocytes nor apolipoprotein B secretion. J Biol Chem 2004;279:23916-23924.
40) Fagerberg CR, Taylor A, Distelmaier F, Schroder HD, Kibaek M, Wieczorek D, et al. Choline transporter-like 1 deficiency causes a new type of childhood-onset neurodegeneration. Brain 2020;143:94-111.
41) Schenkel LC, Bakovic M. Palmitic acid and oleic acid differentially regulate choline transporter-like 1 levels and glycerolipid metabolism in skeletal muscle cells. Lipids 2014;49:731-744.
42) Petkevicius K, Virtue S, Bidault G, Jenkins B, Cubuk C, Morgantini C, et al. Accelerated phosphatidylcholine turnover in macrophages promotes adipose tissue inflammation in obesity. Elife 2019;8:pii:e47990.
43) **Lancaster GI**, **Langley KG**, Berglund NA, Kammoun HL, Reibe S, Estevez E, et al. Evidence that TLR4 is not a receptor for saturated fatty acids but mediates lipid-induced inflammation by reprogramming macrophage metabolism. Cell Metab 2018;27:1096-1110.e1095.
44) **Selathurai A**, **Kowalski GM**, Burch ML, Sepulveda P, Risis S, Lee-Young RS, et al. The CDP-ethanolamine pathway regulates skeletal muscle diacylglycerol content and mitochondrial biogenesis without altering insulin sensitivity. Cell Metab 2015;21:718-730.
45) Fu S, Watkins SM, Hotamisligil GS. The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling. Cell Metab 2012;15:623-634.
46) Quinn WJ 3rd, Wan M, Shewale SV, Gelfer R, Rader DJ, Birnbaum MJ, et al. mTORC1 stimulates phosphatidylcholine synthesis to promote triglyceride secretion. J Clin Invest 2017;127:4207-4215.
47) Lake AD, Novak P, Fisher CD, Jackson JP, Hardwick RN, Billheimer DD, et al. Analysis of global and absorption, distribution, metabolism, and elimination gene expression in the progressive stages of human nonalcoholic fatty liver disease. Drug Metab Dispos 2011;39:1954-1960.
48) da Costa KA, Corbin KD, Niculescu MD, Galanko JA, Zeisel SH. Identification of new genetic polymorphisms that alter the dietary requirement for choline and vary in their distribution across ethnic and racial groups. FASEB J 2014;28:2970-2978.
49) Payne F, Lim K, Girousse A, Brown RJ, Kory N, Robbins A, et al. Mutations disrupting the Kennedy phosphatidylcholine pathway in humans with congenital lipodystrophy and fatty liver disease. Proc Natl Acad Sci U S A 2014;111:8901-8906.

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