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# Choline supplementation restores substrate balance and alleviates complications of Pcyt2 deficiency

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**Abstract**

Choline plays a critical role in systemic lipid metabolism and hepatic function. Here we conducted a series of experiments to investigate the effect of choline supplementation on metabolically altered Pcyt2$^{+/-}$ mice. In Pcyt2$^{+/-}$ mice, the membrane phosphatidylethanolamine (PE) turnover is reduced and the formation of fatty acids (FA) and triglycerides (TAG) increased, resulting in hypertriglyceridemia, liver steatosis and obesity. One month of choline supplementation reduced the incorporation of FA into TAG and facilitated TAG degradation in Pcyt2$^{+/-}$ adipocytes, plasma and liver. Choline particularly stimulated adipocyte and liver TAG lipolysis by specific lipases (ATGL, LPL and HSL) and inhibited TAG formation by DGAT1 and DGAT2. Choline also activated the liver AMPK and mitochondrial FA oxidation gene PPARα and reduced the FA synthesis genes SREBP1, SCD1 and FAS. Liver (HPLC) and plasma (tandem mass spectroscopy and $^1$H-NMR) metabolite profiling established that Pcyt2$^{+/-}$ mice have reduced membrane cholesterol/sphingomyelin ratio and the homocysteine/methionine cycle that were improved by choline supplementation. These data suggest that supplementary choline is beneficial for restoring FA and TAG homeostasis under conditions of obesity caused by impaired PE synthesis.
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*Keywords:* Pcyt2; Choline; Ethanolamine; Kennedy pathway; Phospholipids

## 1. Introduction

Choline is considered an essential nutrient for both humans and animals [1]. It is a precursor for the synthesis of the major phospholipid phosphatidylcholine (PC), of the neurotransmitter acetylcholine and of betaine, an important methyl group donor. Choline also plays a critical role in systemic lipid metabolism and hepatic and muscle function [2–5]. Choline homeostasis is regulated by metabolism and by multiple protein-mediated transport systems in and out of the cells. The choline transporter-like protein 1 (CTL1/SLC44A1) transports choline in the plasma membrane and mitochondria [6–8] and is considered the most important choline transporter for *de novo* PC biosynthesis by the Kennedy pathway [6,8–10]. There are two branches of this pathway, the CDP-choline pathway for the biosynthesis of PC and the CDP-ethanolamine pathway for the biosynthesis of phosphatidylethanolamine (PE). The two pathways share the enzymes, choline/ethanolamine kinase and choline/ethanolamine phosphotransferase, as well as the substrates ATP, CTP and diacylglycerol (DAG) [11,12]. Functional distinction between the two pathways is mainly at the level of formation of CDP-choline and CDP-ethanolamine governed by CTP:cholinephophate (CT/Pcyt1) and CTP:ethanolaminephosphate (ET/Pcyt2) cytidylyltransferases. PC and PE are also metabolically connected through biosynthesis of phosphatidylserine (PS). They are substrates for the formation of PS in the base-exchange reactions catalyzed by PS synthases, PSS1 for PC and PSS2 for PE. The newly formed PS contributes the membrane bilayers or could be transformed into PE (but not PC) by mitochondrial PS decarboxylase (PSD). Mostly in the liver, PE could be converted into PC in a three-step methylation reaction catalyzed by PE methyltransferase (PEMT).

The CDP-choline pathway, as well as PSD, PSS and PEMT reactions, has been extensively characterized [13–16]. Strangely enough, there is no information available on how the impairments in the CDP-ethanolamine pathway impact the CDP-choline pathway and PC catabolism. Our group has developed a Pcyt2 heterozygous mice (HET) in which the flux through the CDP-ethanolamine pathway is reduced [17]. These mice also have reduced PE turnover and increased fatty acid (FA) and triacylglycerol (TAG) formation, and they develop adult-onset obesity, dyslipidemia and fatty liver and insulin resistance, all well-known characteristics of the metabolic syndrome [17,18]. We demonstrated that young HET mice have elevated FA synthesis even before these symptoms appear, to esterify excess DAG unused in the

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CDP-ethanolamine pathway [19]. This process however elevates TAG and leads to obesity and liver steatosis in adults. Thus, HET mouse is a unique model for studying the contribution of defective membrane phospholipid biogenesis to development of metabolic diseases.

Choline is consumed for membrane PC and sphingomyelin (SM) formation and by oxidation to betaine in mitochondria [6,8–10,20]. A previous study has shown that methyl group supplementation, including choline, betaine, folic acid, vitamin B12 and methionine, could reduce fat accumulation in the liver of obese mice induced by high-fat diet [21]. We proposed that stimulation of choline metabolism could be a good strategy to reverse the metabolic complications of Pcyt2 deficiency. We conducted a series of experiments to investigate the effects of choline supplementation on Pcyt2 HET pathologies. We hypothesize that increased choline availability will restore the membrane function and facilitate TAG degradation consequently reducing the obesity phenotype of HET mice.

## 2. Methods

### 2.1. Animals and genotyping

Generation of Pcyt2 deficient ($Pcyt2^{+/-}$) mice was described previously [17]. All procedures were approved by the University of Guelph's Animal Care Committee and were in accordance with guidelines of the Canadian Council on Animal Care. Mice were exposed to a 12-h light/12-h dark cycle beginning with light at 7:00 a.m. Mice were fed a standardized diet (Harlan Teklad S-2335) *ad libitum* and had free access to water.

Pcyt2 genotypes (heterozygous $Pcyt2^{+/-}$ and wild-type $Pcyt2^{+/+}$) were determined from tail genomic DNA. Tissues were digested with 200 μg of proteinase K at 55°C in a buffer [10 mM Tris–HCl (pH 8.0), 0.1 M EDTA (pH 8.0), 0.5% sodium dodecyl sulfate and 0.1 M NaCl]. To eliminate RNA, 10 μg of RNase A was added. Genomic DNA was amplified by PCR using a common upstream primer, FP (CCTGGAACT CATGAGATCCTCCTG), in combination with either a downstream primer, RP (ATCGCACCACACCCGCACGA), specific for the wild-type allele or primer N1 (TGCGAGGCCAGAGGCCACTTGTGTAGC), specific for the knockout allele. PCR was performed with 0.3 units of REDtaq DNA polymerase (Sigma). Initial denaturation was at 94°C for 5 min, followed by 32 cycles at 94°C for 1 min, 60°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 10 min. The primer pairs FP/RP and FP/N1 yielded products of 450 and 305 bp, respectively [17].

### 2.2. Choline dietary treatments

HET mice progressively develop obesity and insulin resistance starting at 2.5 months of age [17]. In the present study, 8- to 10-month-old mice were separated by sexes and divided into three groups ($n$=6–10 in each group): $Pcyt2^{+/+}$ control littermates (WT), $Pcyt2^{+/-}$ (HET) and $Pcyt2^{+/-}$ treated with choline (HET+Cho). HET+Cho mice had access to water containing 2 mg/ml choline (Sigma) for 4 weeks. Water intake was recorded daily and body weight was recorded weekly. Food intake was visually tracked. Mice were euthanized after 4 weeks of choline treatment. Tissues and plasma were collected, immediately used or frozen in liquid nitrogen and stored at −80°C.

### 2.3. Histology analyses

Fresh livers and adipose tissue from WT, HET and HET+Cho females ($n$=4 each group) were fixed in 10% formalin in phosphate-buffered saline (PBS) and embedded in parafilm until out for histopathologic examination. Sections of 10 μm were dewaxed in xylene and rehydrated in ethanol. The sections were stained with hematoxylin and eosin to examine the amount of liver lipid droplets and adipocyte number and cell size. In addition, liver sections were stained with 0.1% Picrosirius red die (Sigma) for collagen content (Ontario Veterinary College, Department of Pathobiology, University of Guelph). Histomorphometric analysis was performed with a computerized image analysis system composed of a photomicroscope and digital camera (Olympus Biological Microscopes: FSX100; Carl Zeiss Microscopy, Oberkochen, Germany) and software (FSX-100; Olympus Global). The entire liver section was captured by consecutive fields at a magnification of ×20 with no overlapping. The mean of the red-stained area of all fields in each section was calculated. The mean area of fibrosis in square micrometers per field was calculated for each liver section. Adipose cell size from WT and HET females was similarly measured using bright-field microscopy.

### 2.4. Plasma triglyceride analysis

Blood samples from WT, HET and HET+Cho female mice ($n$=4 each group) were dispensed into EDTA-coated tubes and stored on ice until ready for centrifugation. Plasma was obtained following centrifugation at 3000g for 10 min at 4°C; 80–100 μl of plasma was transferred into sterilized 1.5-ml Eppendorf tubes and kept at −80°C until analysis. TAG analysis was performed in 96-well plates exactly as described (Sigma TR0100 Kit).

### 2.5. In vivo radiolabeling

The conditions for *in vivo* radiolabeling experiments were as previously described [17] with minor modifications. WT, HET and HET+Cho males ($n$=6 each group) were ip injected with 0.5 μCi (1 μCi/μl) of [$^{14}$C]oleate and 5 μCi (1 μCi/μl) of [$^{3}$H]choline diluted in saline. After 1.5 h, mice were sacrificed and liver samples were collected. The liver lipids were extracted by the Bligh–Dyer method [22] and separated by thin-layer chromatography [4,23]. $^{3}$H-PC and $^{14}$C-PC was separated with chloroform/methanol/acetic acid/water (40:12:2:0.75) and stained with 15% sulfuric acid and 0.5% $K_2CrO_7$. $^{14}$C-DAG and $^{14}$C-TAG were separated with heptane/isopropyl alcohol/acetic acid (60:40:3) and stained with iodine. The radioactivity of lipid compounds was determined by liquid scintillation counting.

### 2.6. Liver lipid analysis

Liver samples (60 mg) were homogenized in 300 μl of 5% PBS-T, the homogenates twice heated to 100°C and cooled to room temperature and centrifuged at 15000 rpm for 2 min to remove insoluble cell debris. TAG content was determined exactly as described (Wako Diagnostics Kit). The analysis of other lipids was performed by HPLC (Agilent Technologies, Santa Clara, CA, USA) as before [24]. Briefly, 1 mg of liver homogenates from WT, HET and HET+Cho mice ($n$=3) was extracted in the presence of an internal standard (50 mg dipalmitoyl-phosphatidyl dimethyl ethanolamine) using a method of Folch *et al.*[25]. The extracted lipids were dried, resuspended in chloroform/isooctane (1:1) and separated by a three-solvent gradient and a normal-phase column (Onyx monolithic silica; Phenomenex Incorporated, Torrance, CA, USA). The amounts of PC, PE, phosphatidylinositol (PI), SM, free cholesterol (FC) and cholesterol esters (CE) were determined using appropriate standards and expressed in micrograms of lipid per milligram of protein.

### 2.7. CTL1 immunoblotting

Frozen liver samples were homogenized in a cold lysis buffer [10 mM Tris–HCl (pH 7.4), 1 mM EDTA and 10 mM NaF] containing protease (1/10) and phosphatase (1/100) inhibitor cocktails (Sigma). The lysate was centrifuged at 2,000g for 20 min at 4°C to remove cell debris, and 15 μg of lysate was mixed with a nondenaturing loading buffer (62 mM Tris–HCl and 0.01% bromophenol blue in 10% glycerol) and separated on a native gel at 110 V for 1.5 h. Proteins were

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