Mechanism of choline deficiency and membrane alteration in postural orthostatic tachycardia syndrome primary skin fibroblasts

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ABSTRACT **Fibroblasts from a patient with postural orthostatic tachycardia syndrome (POTS), who presented with low plasma choline and betaine, were studied to determine the metabolic characteristics of the choline deficiency. Choline is required for the synthesis of the phospholipid phosphatidylcholine (PC) and for betaine, an important osmoregulator. Here, choline transport, lipid homeostasis, and mitochondria function were analyzed in skin fibroblasts from POTS and compared with control cells. The choline transporter-like protein 1/solute carrier 44A1 (CTL1/SLC44A1) and mRNA expression were 2–3 times lower in POTS fibroblasts, and choline uptake was reduced 60% ($P < 0.05$). Disturbances of membrane homeostasis were observed by reduced ratios between PC:phosphatidylethanolamine and sphingomyelin:cholesterol, as well as by modified phospholipid fatty acid composition. Choline deficiency also impaired mitochondria function, which was observed by a reduction in oxygen consumption, mitochondrial potential, and glycolytic activity. When POTS cells were treated with choline, transporter was up-regulated, and uptake of choline increased, offering an option for patient treatment. The characteristics of the POTS fibroblasts described here represent a first model of choline and CTL1/SLC44A1 deficiency, in which choline transport, membrane homeostasis, and mitochondrial function are impaired.—Schenkel, L. C., Singh, R. K., Michel, V., Zeisel, S. H., da Costa, K.-A., Johnson, A. R., Mudd, H. S., Bakovic, M. Mechanism of choline deficiency and membrane alteration in postural orthostatic tachycardia syndrome primary skin fibroblasts. *FASEB J.* 29, 1663–1675 (2015). www.fasebj.org**

Key Words: choline transport · CTL1/SLC44A1 · mitochondria · phospholipids

POSTURAL ORTHOSTATIC tachycardia syndrome (POTS) is characterized by orthostatic intolerance accompanied by cerebral hypoperfusion and sympathetic hyperactivity. Several studies indicate that POTS is pathophysiologically heterogeneous and frequently presents multiple chronic symptoms (1). The cause of this condition is still unclear. Many patients with POTS have hypovolemia and require volume expansion with adequate daily water and sodium intake or pharmacologic therapy to promote intravascular volume expansion or peripheral vasoconstriction. Here, we studied primary skin cells from a 28-yr-old female diagnosed with POTS who presented with very low concentrations of plasma choline and betaine. A possible connection between these observations may be the roles of betaine and glycerophosphocholine as osmolytes, which regulate blood pressure (2), as well as the role of choline in phosphatidylcholine (PC) formation, membrane fluidity, and integrity (3, 4).

Intracellular choline availability and metabolism are associated with lipid homeostasis (5–7). Choline is required for the biosynthesis of the major membrane phospholipid PC, which plays a major role in cellular proliferation, membrane fluidity and functions, and lipid metabolism (8). The majority of PC synthesis occurs *via* the cytidine 5′-diphosphocholine (CDP-choline) or Kennedy pathway (8, 9). The transport of choline into the cell for PC synthesis is regulated by the choline transporter-like protein 1/solute carrier 44A1 (CTL1/SLC44A1) in the plasma membrane (10). Immediately after entering the cells, choline is phosphorylated by choline kinases (11). The kinase product phosphocholine is then coupled with CTP by the regulatory pathway enzyme CTP:phosphocholine-cytidylyltransferase

Abbreviations: AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; BCA, bicinchoninic acid; BHMT, betaine homocysteine methyltransferase; CCT/Pcyt1, CTP:phosphocholine-cytidylyltransferase; CDP-choline, cytidine 5′-diphosphocholine; CL, cardiolipin; CTL1/SLC44A1, choline transporter-like protein 1/solute carrier 44A1; DAG, diacylglycerol; DCF, dihydrodichlorofluorescein diacetate; ECAR, extracellular acidification rate; ESI, electrospray ionization; FC, free cholesterol;

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(CCT/Pcyt1) to yield CDP-choline and to release inorganic pyrophosphate. In the final step, the CDP-choline derivative is condensed with diacylglycerol (DAG), catalyzed by multiple DAG:choline and DAG:ethanolamine phosphotransferases, to release CDP and to produce the bilayer forming phospholipid PC at the endoplasmic reticulum. In the liver, an alternative pathway utilizes phosphatidylethanolamine (PE) to produce PC in a 3-step methylation of PE by S-adenosylmethionine (AdoMet) catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT) (12). The newly synthesized PC can be converted to other phospholipids: PC can be metabolized to generate phosphatidylserine (PS) by the exchange of serine for the choline head groups of PC; PS can then be decarboxylated to PE by PS decarboxylase. In addition, PC metabolizes to glycerophosphocholine, a major osmolyte derived from choline. The PC catabolism is mainly catalyzed by nonlysosomal phospholipases. These phospholipases are divided into 3 classes based on the bond they cleave: phospholipase A (PLA1 and PLA2), PLC, and phospholipase D (PLD). PLD hydrolyzes PC to form phosphatidic acid (PA) and regenerates choline. The latter, in turn, can re-enter the Kennedy pathway or be converted to betaine.

In the liver and kidney mitochondria, choline is oxidized to betaine in a 2-step reaction catalyzed by choline dehydrogenase on the inner side of the inner mitochondrial membrane and by betaine aldehyde dehydrogenase in the mitochondrial matrix (13). The availability of choline in the mitochondria, and thus the rate of choline oxidation, is controlled by protein-mediated choline transport through mitochondrial membranes. In the outer mitochondrial membrane, CTL1/SLC44A1 is characterized as a choline transporter and regulates the choline uptake into mitochondria (14). An inner mitochondrial membrane transporter has been shown to participate in choline oxidation to betaine; however, its structural identity is still unknown (15). Betaine is a methyl group donor that can enter the 1-carbon metabolism and remethylate homocysteine (tHcy) to methionine, a reaction catalyzed by betaine homocysteine methyltransferase (BHMT). The transfer of adenosine to methionine then yields AdoMet, which is the source of methyl groups for numerous methyltransferases, such as PEMT. Besides its role as a methyl group donor, betaine is an important organic osmolyte that helps to maintain cell volume and hydration of cellular proteins in the kidney (16, 17).

The low plasma choline observed in this patient with POTS may be an indicator of altered lipid homeostasis, membrane integrity, and cell function. Here, we describe the results of a series of experiments designed to characterize the transport and metabolism of choline and the regulation of phospholipids in primary skin cells isolated from the patient with POTS in order to investigate why plasma choline concentration was so low.

MATERIALS AND METHODS

Blood metabolites

The patient with POTS fasted overnight (at least 12 h) before blood was drawn by venipuncture into an EDTA tube, which was immediately placed on ice and spun in the cold to separate plasma for analysis, or a serum separator tube to collect serum after clotting. Choline, PC, and betaine were extracted from plasma by the method of Bligh and Dyer (18). Aqueous and organic compounds were separated, analyzed, and quantified directly by liquid chromatography/electrospray ionization (ESI)-isotope dilution mass spectrometry after the addition of internal standards (ISs) labeled with stable isotopes to correct for recovery (19). A validated control plasma that was collected, processed, and stored under the same conditions as the patient was run every time POTS plasma was analyzed. AdoMet and S-adenosylhomocysteine (AdoHcy) in plasma were measured by HPLC with fluorescence detection after conversion into their fluorescent isoindoles (20). These measurements were done by Dr. Conrad Wagner at Vanderbilt University (Nashville, TN, USA).

Methionine, tHcy, and 15 other metabolites in **Table 1** were measured in serum with the use of a capillary GC-MS method previously described (21). These measurements were done by Dr. Sally Stabler (University of Colorado, Aurora, CO, USA). Briefly, serum samples were extracted, and metabolites were isolated with the use of an anion-exchange column, derivatized with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide, and

TABLE 1. *Metabolites measured from the patient with POTS*

Metabolite	Physiologic range	POTS
Methionine	13.3–42.7 μM	29.2
AdoMet	92.8 ± 16.2 nM	123
AdoHcy	27.8 ± 7.9 nM	21.9
AdoMet/AdoHcy	2.1–7.0	5.6
tHcy	5.1–13.9 μM	34.3
Cystathionine	44–342 nM	468
Cysteine	203–369 μM	172
Sarcosine	0.6–2.67 μM	0.88
PC	1401–3001 μM[a]	2131
Choline	6.4–15.6 μM[a]	4.7
Betaine	26–145 μM[a]	10.2
Dimethylglycine	1.4–5.3 μM	4.49
Methylmalonate	73–271 μM	253
2-Methylcitrate	60–228 μM	102
Glycine	152–413 μM	163
Serine	97–267 μM	61
α-Aminobutyrate	8–30 μM	6

Plasma or serum was isolated from blood collected in the fasted state, and metabolites were analyzed by the chromatography assay described in Materials and Methods. [a]Physiologic range was determined from 86 men and women receiving 550 mg choline/70 kg body weight per day (adequate intake level), with average values of 10, 61, and 1828 μM for choline, betaine, and PC, respectively.

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FCCP, carbonyl cyanide p-(triflurometho xy-) phenyl-hydrazone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IS, internal standard; LSC, liquid scintillation counting; MEM, minimum essential medium; mtDNA, mitochondrial DNA; OCR, oxygen consumption rate; PA, phosphatidic acid; PC, phosphatidylcholine; PCho, P-choline; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; PI, phosphatydylinositol; PLD, phospholipase D; POTS, postural orthostatic tachycardia syndrome; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SM, sphingomyelin; TAG, triacylglycerol; TBS-T, Tris-buffered saline Tween 20; tHcy, homocysteine; TLC, thin-layer chromatography

analyzed by mass spectrometry. Deuterated standards were used to correct for recovery.

Isolation and maintenance of primary POTS cells

Skin biopsy tissues from the patient with POTS were minced into small pieces and placed in a tissue-cultured flask with minimum essential medium (MEM; containing 1 mg/L choline chloride) supplemented with 20% fetal bovine serum and 1% antibiotic-antimycotic mixture. Flasks were kept in a humidified atmosphere at 37°C and 5% CO_2. After 3 wk, fibroblast cells began to grow out from the skin pieces, and once they reached confluency, cells were trypsinized and transferred to a new flask. Cells were kept in culture for approximately 10 d before passage, and media were changed every 3 d. An aliquot of cells was kept frozen in liquid nitrogen. Cell lines from 2 unrelated healthy individuals with a similar background were purchased from the Coriell Institute's cell repositories (catalog #GM02674; Camden, NJ, USA) and maintained under the same conditions. These fibroblasts were used as control samples for comparison with those from the patient with POTS.

Cell treatments with excess choline

Fibroblasts from the patient with POTS and controls were grown for 30 d with 2.5 and 5 mg/L choline chloride (Sigma-Aldrich, Oakville, ON, Canada) added to the MEMs above and compared with POTS cells and the 2 control cell lines grown without choline added. During 1 mo of choline treatment, the cells were passaged every 8–10 d. The cell number was monitored by counting, and no differences in cell number and growth rate were observed. After treatment, cells were washed with PBS, protein content was determined, and lysate was used for protein expression and choline uptake studies.

Immunoblotting

Fibroblasts were lysed in a lysis buffer (25 mM Tris, 15% glycerol, 1% Triton X-100, 8 mM $MgCl_2$, 1 mM DTT, protease, and phosphatase inhibitor cocktail) on ice for 5 min and scraped into 1.5 ml tubes. Cell debris was removed by centrifugation at 18,500 *g* for 2 min at 4°C. Protein concentration was determined with bicinchoninic acid (BCA; Pierce, Rockford, IL, USA). The ENS-627 antibody was previously developed by M.B's lab and was shown to detect the 72 kDa size CTL1/SLC44A1 protein under nondenaturing conditions (14). Briefly, samples were mixed with nondenaturing loading buffer (62 mM Tris-HCl, 0.01% bromophenol blue, and 10% glycerol) and separated by PAGE at 120 V for 1.5 h. Proteins were transferred to PVDF membranes (Roche, Indianapolis, IN, USA) and stained with Ponceau S. Membranes were blocked in 5% skim milk in Tris-buffered saline Tween 20 (TBS-T) and then incubated with the CTL1/SLC44A1 antibody ENS-627 (1:100 in 5% skim milk in TBS-T) overnight at 4°C (14). Membranes were washed with TBS-T and then incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000 in 5% skim milk in TBS-T) for 1 h. Membranes were washed in TBS-T, and proteins were visualized using a chemiluminescent substrate (Fisher Scientific, Ottawa, ON, Canada). The amount of cell β-tubulin was determined in parallel and used as a loading control.

RNA isolation and RT-PCR

Total RNA from POTS and control skin cells was isolated with TRIzol (Invitrogen, Life Technologies Incorporated, Burlington, ON, Canada). DNase I was used to eliminate genomic DNA, and cDNA was then synthesized from 2 μg RNA using SuperScript II Reverse Transcriptase (Invitrogen, Life Technologies Incorporated). Expression of CTL1/SLC44A1 mRNA was determined by PCR using the primers described in **Table 2**. PCR conditions were 94°C for 5 min, 32 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and final extension was for 5 min at 72°C. Expression of several genes involved in phospholipid (*PCYT1*, *PCYT2*, *PLD1*, and *PLD2*), triglyceride, and fatty acid (*DGAT1*, *DGAT2*, *LIPIN1*, *FAS*, and *SREBP1*) metabolism was also investigated. PCR conditions were similar to the one described above, with annealing temperature individually adjusted for each set of primers (Table 2). Reactions were standardized by amplifying 18S RNA or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and relative band density was quantified using ImageJ software (NIH, Bethesda, MD, USA).

Isolation of mitochondria

Crude mitochondria were isolated from POTS and control cells according to standard protocols for differential centrifugation (14). Briefly, cells were incubated 10 min with RSB buffer (10 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl) and homogenized. Then, MS buffer (210 nM mannitol, 70 mM sucrose, 5 nM Tris-HCl, and 1 mM EDTA) was added, and the homogenate was centrifuged at 590 *g* for 10 min at 4°C. The final supernatant was centrifuged at 14,700 *g* for 20 min at 4°C, and the resulting pellet (crude mitochondrial fraction) was resuspended in the MS buffer; 20 μg mitochondrial protein was analyzed for CTL1/SLC44A1 expression by immunoblotting as described above. Crude mitochondrial fractions were also used for choline uptake assays and mitochondrial potential.

Choline uptake in whole cells and mitochondria

Choline uptake was measured according to our previously standardized protocols (14, 22, 23). Briefly, cells were washed with KRH buffer [130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), and 10 mM glucose] and incubated with 1 μCi methyl [^{3}H]choline (American Radiolabeled Chemicals, St. Louis, MO, USA) for 5 min at room temperature. Cells were then washed in ice-cold KRH buffer containing 1 mM "cold" choline, to stop the uptake and remove the radiolabeled choline from the cell surface, lysed in 500 μl ice-cold lysis buffer (10 mM Tris-HCl, 1 mM EDTA, and 10 mM NaF), scraped into 1.5 ml tubes, and [^{3}H]choline was analyzed by liquid scintillation counting (LSC). For mitochondrial choline transport, crude mitochondria were suspended in a mitochondrial uptake buffer [120 μM KCl, 5 μM HEPES/potassium hydroxide, 1 μM EGTA, 5 μM KH_2PO_4, 0.5 μM $MgCl_2$, 5 μM L-glutamate, and 1.2 μM L-malate (pH 7.2)] and incubated with 1 μCi methyl [^{3}H]choline for 5 min at room temperature. Mitochondria were then pelleted by centrifugation and washed with the uptake buffer containing an excess of "cold" choline. Mitochondria were again centrifuged 5 min at 14,700 *g*, resuspended in ice-cold lysis buffer, and [^{3}H]choline was determined with LSC. The rate of choline uptake in the whole cells and mitochondria was calculated as dpm/5 min/mg of protein. Protein was quantified using the BCA protein assay (Pierce).

Pulse and pulse-chase radiolabeling of the CDP-choline pathway

For pulse experiments, POTS and control cells were incubated for 1–3 h with 5 nM [^{3}H]methylcholine (5 μCi; ARC, St. Louis, MO, USA). For pulse-chase experiments, cells were pulsed for 3 h with 5 nM [^{3}H]methylcholine, washed with PBS, and then chased 1–3 h with an excess of unlabeled choline. At each time point,

TABLE 2. *Primers and PCR conditions*

Gene	Sequence	Product size (bp)	Annealing temperature (°C)
CTL1	F: TCAACAGCACCAACTTCTGC	246	55
	R: ACAGGAAGCAATGAGCGACT		
PCYT1	F: CCCTCTTTCCGATGGCCCTT	267	55
	R: TGTTTGGGTTCCCCCAGTCC		
PCYT2	F: ACATCATCGCGGGGCTTACAC	190	53
	R: TGACACACCAGGTCCACCTT		
PLD1	F: CCGTCCAGTGAGTCTGAGCAA	198	Touchdown
	R: GGGCGTGGAGTACCTGTCAA		
PLD2	F: CATCTGCGGGCTTCGTACAC	248	57
	R: TCCGCAGACTCAAGGCAAAC		
DGAT1	F: ACTACCGTGGCATCCTGAAC	289	56
	R: GCTGGGAAACACAGAATGGT		
DGAT2	F: AGTGGGTCCTGTCCTTCCTT	321	55
	R: TCACTTCTGTGGCCTCTGTG		
LPIN1	F: TAGCCGGCCAGGTGTTTGTC	180	55
	R: TGTCAACCACTTTCTCTCGGG		
FAS	F: CTGGCTCAGCACCTCTATCC	252	Touchdown
	R: AACTCCAGGTTGTCCCTGTG		
SREBP1	F: TGCACGACTGTCAGCAGATG	180	Touchdown
	R: ACTGTGGAGGCCAGAGTCTC		
GAPDH	F: ACCACAGTCCATGCCATCAC	452	55
	R: TCCACCACCCTGTTGCTGTA		

Expression of genes involved in the phospholipid and fatty acid metabolism was determined in the POTS and control fibroblasts using the information in Table 2. F, forward; R, reverse.

cells were washed with 2× PBS, and total lipids were extracted by the Bligh-Dyer method (18). An aliquot of cell lysates was used to measure the protein concentration. The water-soluble metabolites choline, P-choline (PCho), and CDP-choline were separated in ethanol:water:ammonia (48:95:7) and visualized with phosphomolybdic acid in chloroform-ethanol (1:1) and 1% stannous chloride. The radiolabeled PC was determined from the lipid phase after thin-layer chromatography (TLC) separation with chloroform:methanol:acetic acid:water (40:12:2:0.75) and staining with 15% sulfuric acid and 0.5% K_2CrO_7. Specific bands were scraped from plates, and radioactivity was determined by LSC.

PLD activity assay

The transphosphatidylation assay is a method of measuring PLD activity based on the enzyme's ability to attach ethanol to phospholipid groups, a unique property of this enzyme (24). Confluent cells were prelabeled with 5 μCi/ml [^{3}H]myristic acid (ARC) for 24 h and then incubated with fresh medium containing 0.5% ethanol for 30 min at 37°C. Reactions were terminated with ice-cold methanol, and cells were solubilized in 0.2% SDS and 5 mM EDTA. Protein concentration was determined by the Bradford colorimetric assay for normalization. Lipids were extracted with chloroform:methanol:aqueous SDS:acetic acid (1:2:0.8:0.08 v/v). Chloroform and 0.2 M NaCl were added at a 1:1 ratio to separate the phases, and centrifuged for 5 min. The lower chloroform layer was collected, dried under nitrogen, and redissolved in a chloroform:methanol (2:1) mixture. PA and phosphatiylethanol were separated by TLC using ethyl acetate:isooctane:acetic acid:water (65:15:10:50 v/v) and visualized in iodine vapor. Spots corresponding to PA and phosphatidylethanol were scraped into vials, and the radiolabel was counted. The activity of PLD was quantified from the radioactivity incorporated into phosphatidylethanol and expressed in DPM/24 h incubation.

Measurement of total lipids

HPLC was carried out on an Agilent 1100 instrument equipped with a quaternary pump (Agilent Technologies, Santa Clara, CA, USA) and an Alltech ELSD2000 evaporative light-scattering detector (GenTech, Arcade, NY, USA), using a modified version of the method of Graeve and Janssen (25). Briefly, 1 mg cell homogenates from POTS and control cells was extracted in the presence of IS (50 μg dipalmitoyl-phosphatidyldimethylethanolamine) using a modification of the method of Folch *et al.* (26). The extracted lipid phase was collected, dried, and resuspended in 100 μl chloroform:isooctane (1:1). Lipids were separated using a 3-solvent gradient and a normal-phase HPLC column (Onyx monolithic silica; Phenomenex Incorporated, Torrance, CA, USA). The amounts of phospholipids [PC, PE, PS, and phosphatidylinositol (PI)], triacylglycerol (TAG), sphingomyelin (SM), and free cholesterol (FC) in micrograms of lipid per milligrams of protein were determined using appropriate standards.

Lipidomic analysis

There were 2 pooled samples of POTS and control cells prepared from 2 to 3 independent experiments. The samples were kept at −80°C and sent in nitrogen liquid to a lipidomic facility (University of Toronto, Toronto, ON, Canada). Prior to experiment, cell lysates were thawed and kept at 4°C. After addition of 400 μl PBS, samples were spiked with a mixture of ISs 17:0–14:1 PS, 12:0–13:0 PE, and 17:0–14:1 PC (200 ng). Samples were sonicated for 1 min on ice (550 sonic dismembrator; Fisher Scientific). Volume was adjusted to 1 ml, and lipid extraction was performed according to Bligh and Dyer (18). Organic phases were evaporated to dryness under nitrogen and reconstituted in 500 μl methanol:chloroform (2:1) containing 10 mM ammonium acetate. Mass spectrometry was performed by direct infusion of the lipid extract into an AB Sciex QTRAP 5500 (Framingham, MA, USA) in both positive and negative ESI modes (27, 28). Samples were infused at a constant rate

of 5 μl/min and "T-ed" into the solvent flow of 100 μl/min. Data were acquired by precursor ion and neutral loss scans. Data analysis was performed with LipdView Software (AB Sciex). Results were based on the peak area and expressed as frequency (%) of each species in the total lipid identified.

Mitochondrial potential and mitochondrial DNA content

After isolating crude mitochondria as described above, mitochondrial membrane potential was measured using the Isolated Mitochondria Staining Kit (Sigma-Aldrich). The method uses carbocyanine dye (JC-1), which concentrates in the mitochondrial matrix and results in red fluorescence. A dissipation of potential prevents the accumulation of JC-1 dye and results in green fluorescence. Red fluorescence was read in the FlexStation 3 Microplate Reader fluorimeter (Molecular Devices, Sunnyvale, CA, USA; emission, 590 nm; excitation, 490 nm) using a kinetic method. Valinomycin was used as a dissipating agent and negative control. Results were expressed as a mean fluorescence from 3 independent experiments.

Mitochondrial DNA (mtDNA) content was determined relative to chromosomal DNA. Total DNA was isolated from control and POTS cells using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). PCR was performed for the nuclear β-globin gene (forward primer 5′-aactgggcatgtggagacag-3′ and reverse primer 5′-ccaggccatcactaaaggca-3′) and mtDNA (forward primer 5′-cgaaaggacaagagaaataagg-3′ and reverse primer 5′-ctgtaaagttttaagttttatgcg-3′), using the following conditions: 94°C for 5 min, 32 cycles of 30 s at 94°C, 30 s at 53°C (mtDNA) or 59°C (β-globin), and 30 s at 72°C, and final extension was for 5 min at 72°C. The band density was measured using ImageJ. The mtDNA:β-globin cDNA ratio was used to determine mtDNA differences in POTS and control cells.

Oxygen consumption and extracellular acidification rates

POTS and control cells were seeded in a 24-well plate and incubated until they reached 100% confluence. The fully confluent cells were washed with bicarbonate-free DMEM containing 25 mM glucose and equilibrated at 37°C in a non-CO_2 incubator for 1 h. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), a measure of glycolytic pathway activity, were determined simultaneously using an XF-24 Seahorse Bioscience Extracellular Flux Analyzer (Billerica, MA, USA). In addition to baseline measurements, changes in OCR and ECAR in response to various mitochondrial inhibitors were determined. Cells were treated with the ATPase inhibitor oligomycin (1 μg/ml), followed by the mitochondrial uncoupler carbonyl cyanide p-(trifluromethoxy-) phenyl-hydrazone (FCCP; 4 μM), and finally by the cytochrome *c* reductase (complex III) inhibitor antimycin A (1.5 μg/ml).

Measurement of total mitochondrial dehydrogenase activity by MTT assay

Cells were cultured in a 96-well plate and incubated overnight to allow for attachment. An MTT assay was performed using a CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Measurements were replicated 10 times per culture and the mean data presented.

Reactive oxygen species production

POTS and control fibroblasts (10^5 cells) were seeded into a 6-well plate and incubated overnight to allow for attachment. Reactive oxygen species (ROS) production was measured using the fluorescent probe dihydrodichlorofluorescein diacetate (DCF) following the protocol described by D'Alessandro *et al.* (29). Data presented are the mean values for each culture replicated 6 times.

Statistical analysis

All experiments were replicated in at least 3 independent experiments, except for lipidomic analysis, which was performed in duplicates. One-way ANOVA was performed to compare means between POTS and 2 different control cells (multiple groups), whereas unpaired Student's *t* test was used to compare data between POTS and control or treated *vs.* untreated groups. A P value ≤ 0.05 at 95% confidence interval was considered significant. All statistical tests were performed with GraphPad Prism 4 software (La Jolla, CA, USA).

RESULTS

Blood metabolites are altered in the patient with POTS

The patient symptoms at the age of her diagnosis included trouble maintaining normal heart rate upon standing up and with mild exercise, or even basic activities such as walking up a flight of stairs, which caused tachycardia, significant shortness of breath, and at times angina. Several episodes of presyncope were reported. At the time of skin biopsies, the patient with POTS was on potassium gluconate 595 mg, Wellbutrin 150 mg, and Loestrin 24. Analysis of serum metabolites showed that the patient with POTS had very low circulating choline and betaine (Table 1), as observed in patients fed a low-choline diet (30), as well as high tHcy (Table 1). These values are significantly lower than the validated control plasma, which measured 9 and 48 μM for choline and betaine, respectively. In addition, when the patient with POTS consumed an additional 750 mg choline/d, plasma choline and betaine concentrations did not rise appreciably as would be expected. Betaine, which is the oxidation product of choline, donates its methyl group to tHcy, *via* BHMT to form methionine, which then produces the main methylation agent AdoMet. In the liver, AdoMet methylates PE to PC catalyzed by PEMT, and this reaction produces the majority of circulating tHcy and represents the major link between choline and methionine metabolism (12, 31). In POTS serum, tHcy and its metabolite cystathione were found to be elevated; however, cysteine, a metabolite of cystathione, was reduced (Table 1). Studies have shown that plasma concentrations of betaine and tHcy are inversely correlated (32). The AdoMet:AdoHcy ratio, which was not affected in the patient serum, is typically independent of fluctuations in the BHMT activity (33). Therefore, the patient metabolite profile strongly suggested that the elevated tHcy could be reflecting deficiencies in choline and betaine. Both the lack of betaine to methylate tHcy as well as an increase in AdoMet utilization to produce PC from PE by the PEMT pathway may increase tHcy levels in the serum. In the choline-deficient state, the utilization of AdoMet to synthesize PC from PE in the liver is increased, increasing liver AdoHcy (34), which may result in increased circulating tHcy. Because the patient with POTS did not show reduced circulating PC, but lower choline, the synthesis of PC by the PEMT pathway may be increased to compensate for the lack of choline for

the CDP-choline pathway. Up-regulation of the hepatic PEMT pathway may also contribute to the depletion of betaine observed in the POTS serum.

CTL1/SLC44A1 expression and choline transport are reduced in POTS cells

Because patient plasma methionine, AdoMet, and PC were normal, we postulated that a reduced choline transport for phospholipid synthesis could be causing choline and betaine deficiency. Choline transport outside of neuronal-specific choline transporter CHT is largely mediated by CTL1/SLC44A1 (9). CTL1/SLC44A1 is a ubiquitous choline transporter (10) and mediates the cellular uptake of choline required for membrane phospholipid (PC and SM) synthesis as well as mitochondrial choline uptake to store choline in most cells or to use as a source for betaine in the liver and kidney cells (14). We first analyzed expression of various choline transporters, including CTL1/SLC44A1, in POTS cells. Differently from other transporters such as CTL2 and OCT1/3 (data not shown), CTL1/SLC44A1 was the only choline transporter modified in POTS (**Fig. 1**). Although the level of control β-tubulin was unchanged, the CTL1/SLC44A1 protein was reduced by a factor of ~2.5 in POTS compared to 2 controls of human skin fibroblasts (Fig. 1*A*, *B*). Because the 2 control cell lines had identical levels of CTL1/SLC44A1, we decided to perform the following experiments using only 1 control. CTL1/SLC44A1 mRNA was reduced by a factor of ~3 in POTS cells compared to control cells (Fig. 1*C*, *D*). Because we established that the amount of CTL1/SLC44A1 transporter in POTS was mainly regulated at the transcript level, we also sequenced the CTL1/SLC44A1 regulatory promoter region. However, we did not find any mutation in the CTL1/SLC44A1 gene promoter (data not shown), so the reduced CTL1/SLC44A1 mRNA in POTS may be caused by other factors. Next, we examined whether choline uptake into the cell and into isolated mitochondria was altered in POTS. Indeed, in POTS cells, cellular choline uptake was reduced by 60% (**Fig. 2*A***), and mitochondrial choline transport was reduced by 50% (Fig. 2*B*). Taken together, we showed that choline uptake into the cell as well as into the mitochondria was reduced in the POTS cells, which was accompanied with reduction in the amount of CTL1/SLC44A1 transporter. Interestingly, choline supplementation in the cell culture medium increased the amount of choline transporter (significantly after 5 mg/L; Fig. 1*B*) and choline uptake (at both 2.5 and 5 mg/L choline) into deficient POTS fibroblasts (Fig. 2*C*). The increase in choline uptake after 2.5 mg/L choline likely resulted from translocation of CTL1/SLC44A1 from intracellular compartments to the plasma membrane because the total CTL1/SLC44A1 content did not change significantly. Choline supplementation of control fibroblasts did not affect CTL1/SLC44A1 content and choline uptake.

Choline incorporation into the CDP-choline pathway is reduced, but PC synthesis is not affected in POTS

Because choline uptake and CTL1/SLC44A1 transporter were reduced in POTS cells, we also investigated if the CDP-choline pathway for PC synthesis was specifically affected. To measure the flux through the CDP-choline pathway, we radiolabeled POTS and 2 control cell lines with [^{3}H]choline for 1, 2, and 3 h and measured the incorporation of the radiolabeled choline into various intermediates as a function of time (**Fig. 3**). The radiolabeling data for the pathway intermediates choline, PCho, and CDP-choline showed that the choline incorporation at all 3 steps of the pathway was generally reduced in POTS cells (Fig. 3*A*–*C*; $P < 0.05$). The total [^{3}H]choline incorporation into PC was also significantly decreased in POTS cells compared to 2 control cells in all time points (Fig. 3*D*; $P < 0.05$). The total PC synthesis (area under the curve) was lower in POTS cells than in controls (POTS, 7128; control 1, 8821; and control 2, 8316; Fig. 3*D*). These results demonstrated that the reduced radiolabeling in all steps of the Kennedy pathway was observed because of the initially limited uptake of choline in POTS cells.

Choline release from the Kennedy pathway intermediates and PC is increased in POTS cells

To see if the choline release from PC was affected in POTS, we performed [^{3}H]choline pulse-chase radiolabeling experiments to measure PC degradation as well as degradation of the Kennedy pathway intermediates choline, PCho, and CDP-choline. The cells were prelabeled for 3 h with [^{3}H]choline (synthesis step) and "chased" with an excess of unlabeled choline (degradation step) for different time periods. The amounts of remaining metabolites at different time points were plotted, and the extent of degradation estimated from the slopes and area under the curve is shown in **Fig. 4**. [^{3}H]Choline and [^{3}H]PCho decays (curve slopes) were similar in POTS and 2 control cells; however, the total choline labeling (area under the curve) was reduced (Fig. 4*A*, *B*), in agreement with the reduced [^{3}H]choline uptake. On the other hand, the decays of [^{3}H]CDP-choline and [^{3}H]PC were significantly faster in POTS than in control cells (Fig. 4*C*, *D*). Therefore, [^{3}H]CDP-choline and [^{3}H]PC appeared to be more degraded in POTS cells than in controls as an attempt to provide much needed free choline because the uptake of extracellular choline is reduced.

PLD is activated in POTS cells

PLD hydrolyzes PC to yield PA and free choline. We performed a transphosphatidylation assay (attachment of ethanol to phospholipid groups) to measure PLD activity in POTS and control cells (Fig. 4*E*). We found that [^{3}H]myristate incorporated into phosphatidylethanol (DPM/24 h) was 2-fold higher in POTS than in control cells. The amounts of [^{3}H]PA were also increased 1.7-fold in POTS ($P = 0.025$). Thus, we established that the activity of PLD is dramatically increased, and this observation is in agreement with the increased PC degradation in POTS cells (Fig. 4*D*). We suggest that this activation is part of a compensatory mechanism to provide free choline in the state of reduced choline transport and choline deficiency in POTS.

Membrane phospholipid and cholesterol ratio is modified in POTS cells

We next investigated the membrane status in POTS cells. The membrane content of PC, PE, PS, PI, and SM was not

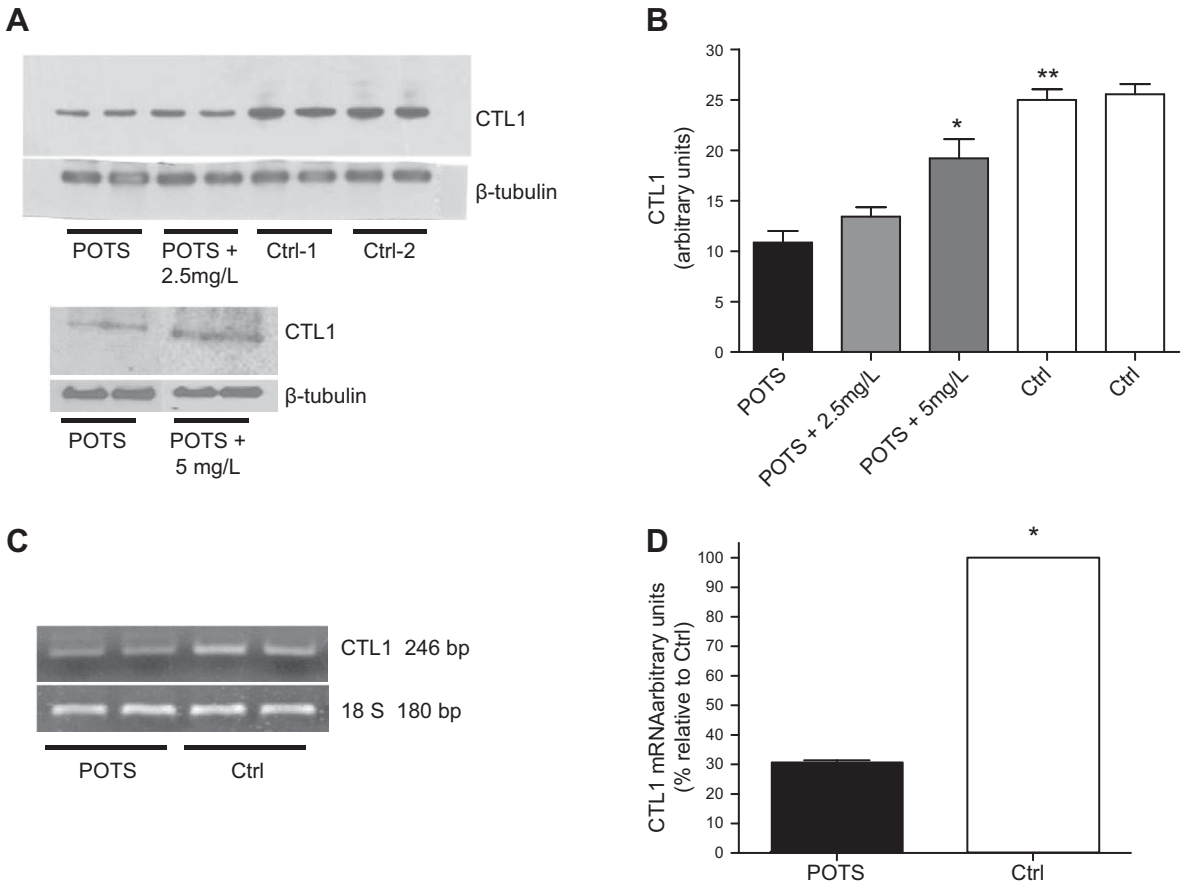

Figure 1. CTL1/SLC44A1 protein and mRNA expression are reduced in POTS cells. *A*) Representative figure of Western blotting for CTL1 in POTS cells untreated and treated with 2.5 and 5 mg/L choline and in 2 control (Ctrl) fibroblasts. *β*-Tubulin was used as loading control and did not differ between groups. *B*) CTL1/SLC44A1 protein was reduced in POTS cells compared to controls. Treatments with 5 mg/L choline significantly increased CTL1/SLC44A1 content in POTS cells. *C*) Representative figure of RT-PCR for CTL1 and 18S in POTS and in control fibroblasts. *D*) CTL1/SLC44A1 mRNA expression was reduced in the POTS compared to control fibroblasts. The 18S loading control did not differ between groups. The intensity of CTL1/SLC44A1 bands was quantified using ImageJ software and expressed as arbitrary units. All experiments were performed in triplicate. $*P <$ 0.05 compared to the untreated patient.

significantly different in POTS compared to control cells (**Fig. 5*A***). However, the ratio of the bilayer forming phospholipids PC and PE was significantly reduced in POTS cells compared to control cells (1.034 ± 0.02 and 1.327 ± 0.14, respectively; $P < 0.05$). The PC:PE ratio is positively correlated with membrane integrity and cellular and mitochondrial function (3, 35). The ratio of SM and FC, which constitute the lipid rafts, was 1.69-fold higher in POTS cells compared to control cells (0.145 ± 0.02 and 0.086 ± 0.02, respectively; $P < 0.05$). The total FC and TAG content was also significantly lower in POTS cells ($P < 0.05$; Fig. 5*B*). These results demonstrated that the POTS membrane bilayer and lipid rafts were severely altered, strongly contributing to modified membrane permeability and function.

The main lipogenic genes are not modified in POTS cells

The Kennedy pathway regulatory genes Pcyt1 (for PC) and Pcyt2 (for PE) were modestly increased by 17% ($P < 0.05$) and 21% ($P < 0.05$), respectively. The expression of PC and PE degradation genes PLD1 and PLD2 was unchanged (**Fig. 6*A, B***), even though the total PLD activity was elevated in POTS cells (Fig. 4*E*). Although total TAG was markedly reduced, the expression of the lipogenic genes (LPIN1, SREBP1, FAS, and DGAT2) was not modified in the POTS cells. DGAT1 expression was however modestly increased (20%; $P < 0.05$), and it was the only compensatory change observed in the lipogenic pathways (Fig. 6*A*, *B*). These data showed that the main regulatory phospholipid and lipogenic genes were not responsible for the severely compromised membrane lipid composition in POTS cells.

Lipidomic analysis

The fatty acid composition of phospholipids [PC, PE, PS, SM, and cardiolipin (CL)] and neutral lipids (DAG and TAG) was performed by "shotgun" lipidomics on pooled samples of POTS and control cells. POTS cells had a lower percentage of long-chain polyunsaturated fatty acid (PUFA) PC (37:4, 37:5, 37:6, 39:5, 39:6, and 40:6) and

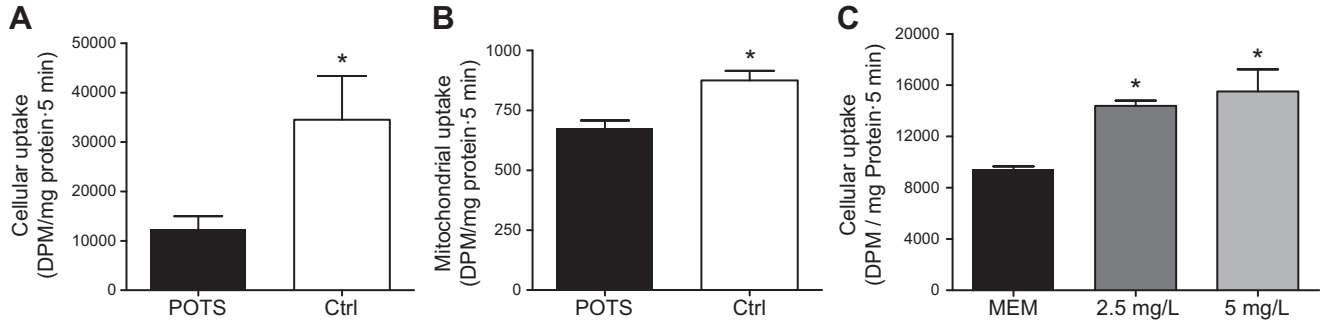

Figure 2. Choline uptake is reduced in POTS cells. Choline uptake was measured using [^{3}H]choline in intact cells and isolated mitochondria. Choline uptake into the cell (*A*) and into the isolated mitochondria (*B*) was reduced in POTS compared to control fibroblasts. *C*) Choline uptake into the POTS cells was increased after treatment with choline at 2.5 and 5 mg/L compared to untreated (MEM) cells. Results are expressed as DPM/mg protein × 5 min. All experiments were performed in triplicate. *$P < 0.05$.

a higher percentage of medium-chain saturated and mono/diunsaturated PC (32:0, 34:1, 34:2, and 36:3) (Supplemental Fig. 1*A*). The opposite trend was observed for PE species. The frequency of most long-chain PUFA-derived PE (36:4, 38:6, 40:4, and 40:5) was higher in POTS cells, whereas the percentage of some medium-chain, saturated and mono/diunsaturated PE (36:1 and 36:2) was higher in control cells. PS showed a similar trend as PE, with a higher contribution of long-chain PUFA in the POTS (Supplemental Fig. 1*B, C*).

The fatty acid profile of CL was similar between POTS and control cells, with only CL 76:9 being higher in POTS. SM showed a higher frequency of monounsaturated fatty acids in the POTS similarly to PC (Supplemental Fig. 1*D*, *E*). The frequency of DAG species did not seem to differ between POTS and control cells (Supplemental Fig. 1*F*). Finally, TAG composition was highly different between the 2 groups. In general, POTS showed a higher frequency of medium-chain and saturated fatty acid TAG (46:0, 48:0, and 50:1) and a lower frequency of long-chain PUFA TAG

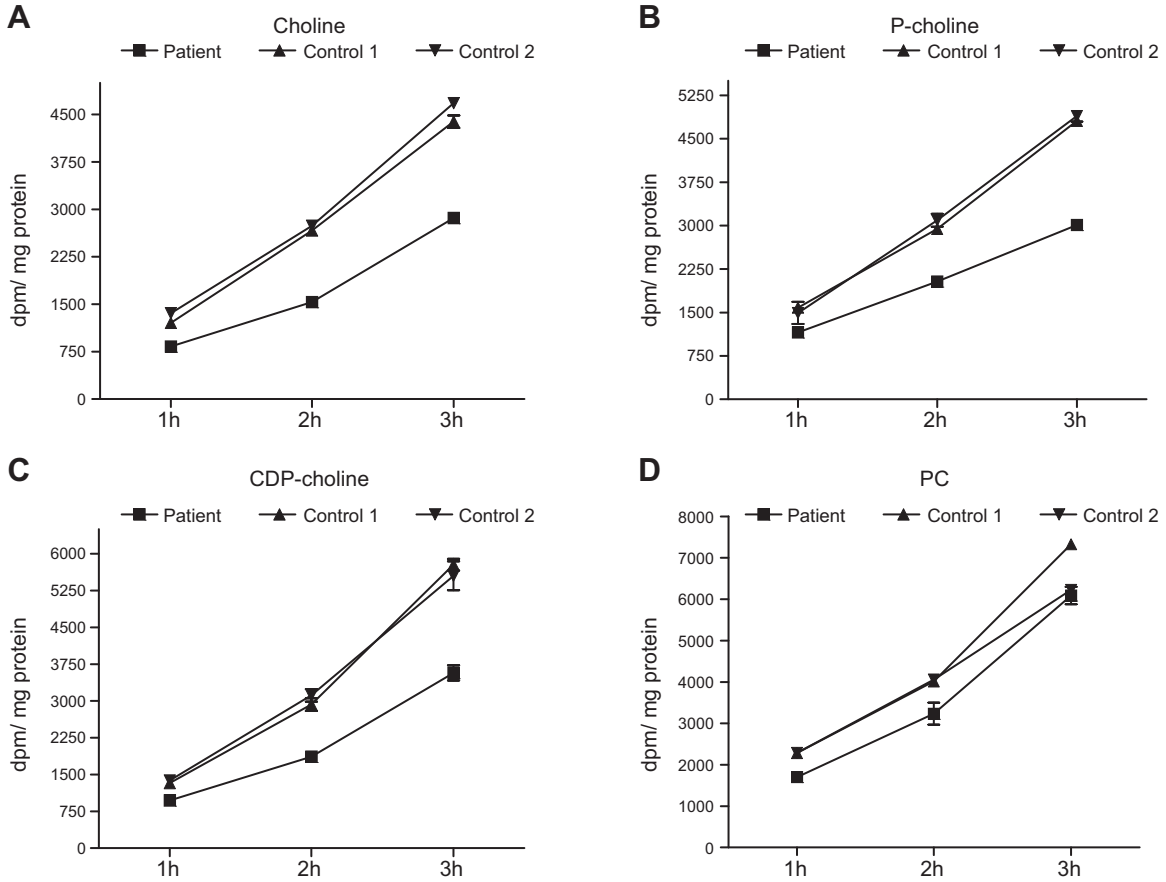

Figure 3. Choline incorporation into CDP-choline pathway intermediates is reduced in POTS cells. Intermediates of the Kennedy pathway and PC synthesis were analyzed after 1, 2, and 3 h pulse with [^{3}H]choline. Because of reduced uptake, POTS fibroblasts showed significantly reduced [^{3}H]choline incorporation (*A*) into PCho (*B*), CDP-choline (*C*), and PC (*D*) compared to 2 control fibroblasts, but the rate of PC synthesis was unchanged. All experiments were performed in triplicate. *$P < 0.05$.

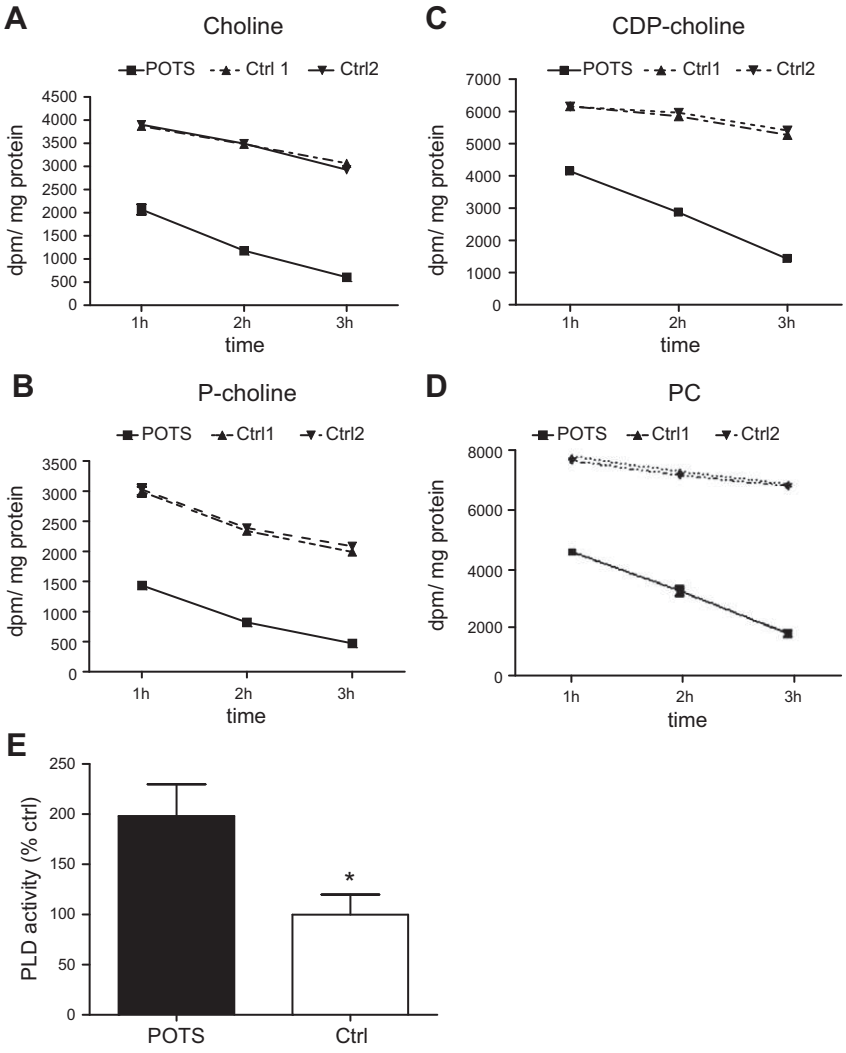

Figure 4. Degradation of the CDP-choline pathway intermediates and PC is increased in POTS cells. The pulse-chase experiment was performed by incubation with [^{3}H] choline for 3 h and chase period of 1, 2, and 3 h. POTS cells showed faster degradation of choline (*A*), PCho (*B*), CDP-choline (*C*), and PC (*D*) compared with 2 control fibroblasts. *E*) PLD activity was increased in POTS fibroblasts compared with control. $*P \leq 0.05$. PLD activity was measured in DPM as the amount of [^{3}H]myristic acid incorporated into phosphatidylethanol. All experiments were performed in triplicate. $*P < 0.05$.

species (56:2, 56:3, 56:4, 58:6, and 60:9) (Supplemental Fig. 1*G*). We concluded that the fatty acid profiles of the major membrane phospholipids and TAG were altered in POTS cells compared to control as a result of broadly modified glycerolipid metabolism and remodeling in POTS cells.

Mitochondrial function is impaired in POTS cells

Because the mitochondrial choline uptake was 50% reduced in the POTS cells (Fig. 2*B*), we further examined the mitochondrial function and membrane potential of these

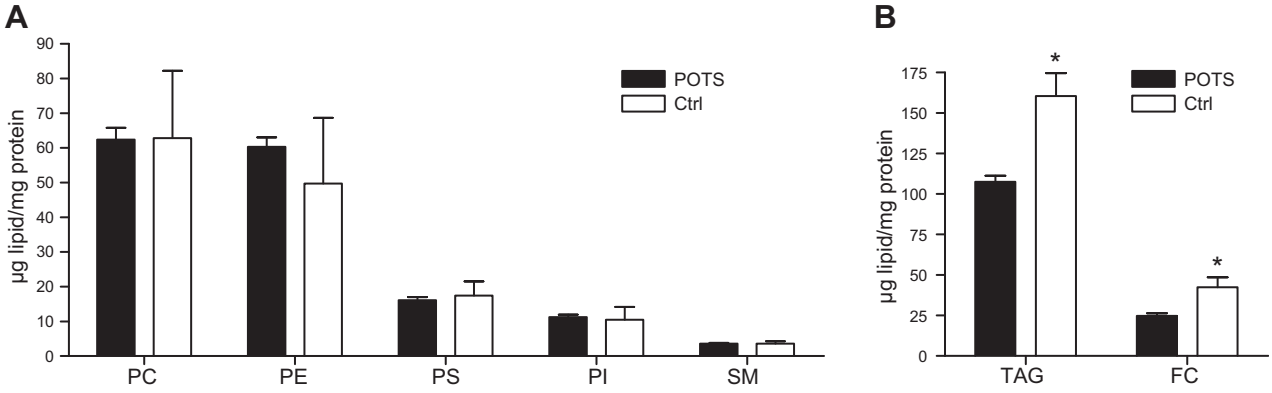

Figure 5. Membrane phospholipid and cholesterol ratio is modified in POTS cells. The total content of phospholipids, cholesterol, and triglycerides was analyzed by HPLC in POTS and control cells. *A*) The total amounts of PC, PE, PS, PI, and SM did not differ between POTS and control cells; however, the ratio between PC:PE and SM:FC was significantly reduced in POTS cells. *B*) Cholesterol (FC) and TAG were significantly lower in POTS compared with control cells. The experiment was performed in triplicate. $*P < 0.05$.

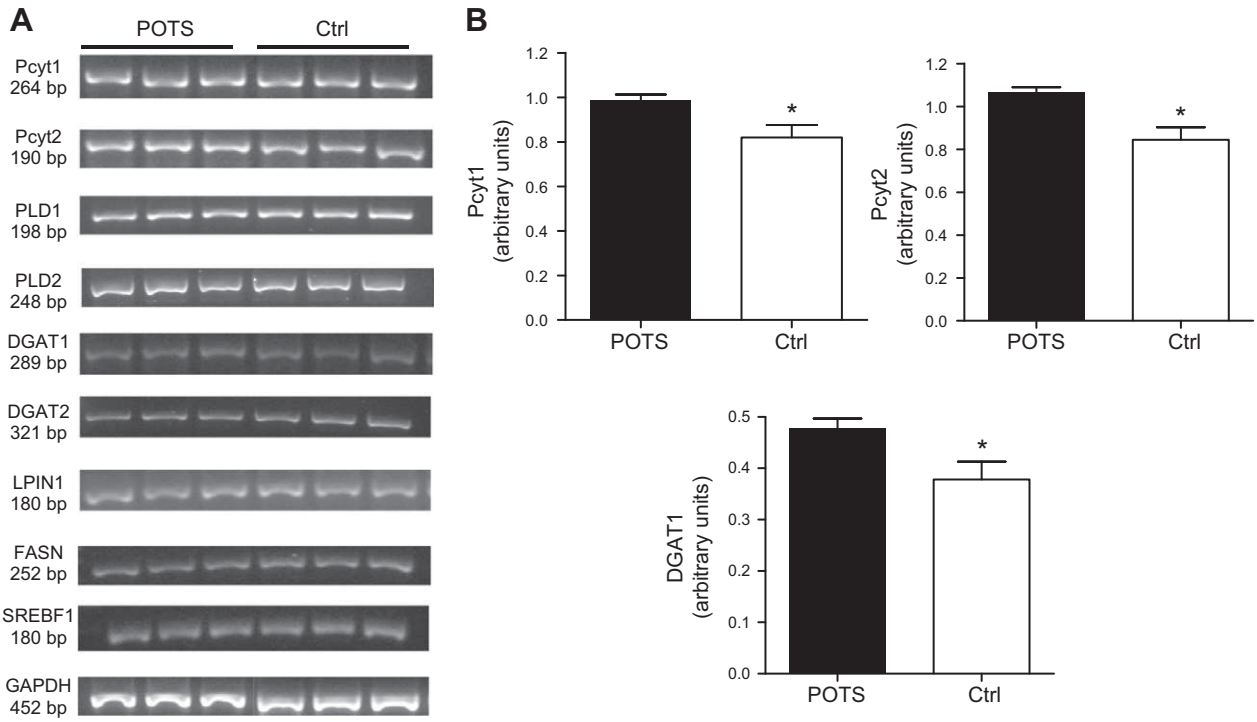

Figure 6. Lypogenic gene expression is not altered in POTS cells. *A*) mRNA expression of lipid regulatory genes analyzed by RT-PCR and stained with SYBR Green. *B*) Pcyt1, Pcyt2, and DGAT1 expression was significantly higher in patient fibroblasts. $*P < 0.05$. PLD1, PLD2, DGAT2, LPIN1, FASN, and SREBF1 expression did not differ between POTS and control fibroblasts. The intensity of PCR bands was quantified using ImageJ software and expressed as arbitrary units. Experiments were performed in triplicate, and results were normalized by the loading control GAPDH.

cells (**Fig. 7**) We found that the mitochondria potential was reduced by a factor of 1.5 in the POTS cells compared to control cells (Fig. 7*A*). Mitochondria potential has been related to choline uptake into the mitochondria (36), and the decrease in the potential observed here may contribute to the decrease in choline transport into the mitochondria in POTS cells.

We then measured the electron transfer rates through the electron transport chain by the OCR. Baseline measures of OCR were almost 3 times lower in POTS fibroblasts than in control fibroblasts (Fig. 7*C*), which could be the result of decreased expression and/or activity of tricarboxylic acid cycle or oxidative phosphorylation proteins, or another anomaly impairing electron transport chain function upstream of complex III. Treatments with oligomycin decreased OCR 65% in control fibroblasts, but only 50% in POTS, indicating more proton leakage across the inner mitochondrial membrane in the POTS cells. Following FCCP treatment, OCR increased 7-fold in control, whereas in POTS, OCR only increased 4-fold. These results clearly indicated that the POTS cells have a decreased respiration capacity compared to controls, and the mitochondria in those cells may be unable to provide adequate amounts of ATP during times of increased energy demand.

The ECAR assay, a measure of glycolytic activity, showed that baseline ECAR was 40% lower in POTS fibroblasts compared to control cells, suggesting a block in the glycolytic pathway (Fig. 7*D*). Inhibition of ATP synthase caused glycolytic rates to increase in both cell cultures, as expected, but the ECAR response to oligomycin was lower in the POTS fibroblasts. POTS cells also produce more ROS compared to controls (Fig. 7*E*). However, total mitochondrial dehydrogenase activity measured by MTT assay was increased in the POTS compared to control fibroblasts (Fig. 7*F*). Therefore, the respiratory capacity of the mitochondria was reduced in the POTS cells. In addition, we showed that the mtDNA copy number is not altered (Fig. 7*B*), so the reduction in the mitochondrial membrane potential and oxidative capacity in POTS cells could not be attributed to a reduced number of mitochondria.

DISCUSSION

This study describes a disorder related to choline deficiency in a female, who was diagnosed with dysautonomia and POTS. The analysis of patient blood showed very low amounts of choline and betaine, which suggested impairments in choline-related phospholipid pathways and choline transport activity. Using cells obtained from the patient's skin biopsies, we clearly established that the reduced cellular and mitochondrial choline uptake and reduced choline transporter CTL1/SLC44A1 were responsible for the observed deficiency. This is the first known case of a medical disorder related to transport-mediated choline deficiency.

Because the lack of sufficient intracellular choline seems to be caused by impaired cellular choline transport, we sought to determine the effect of choline treatment on POTS cells. Choline uptake into the POTS cells was increased after choline treatments in a dose-dependent

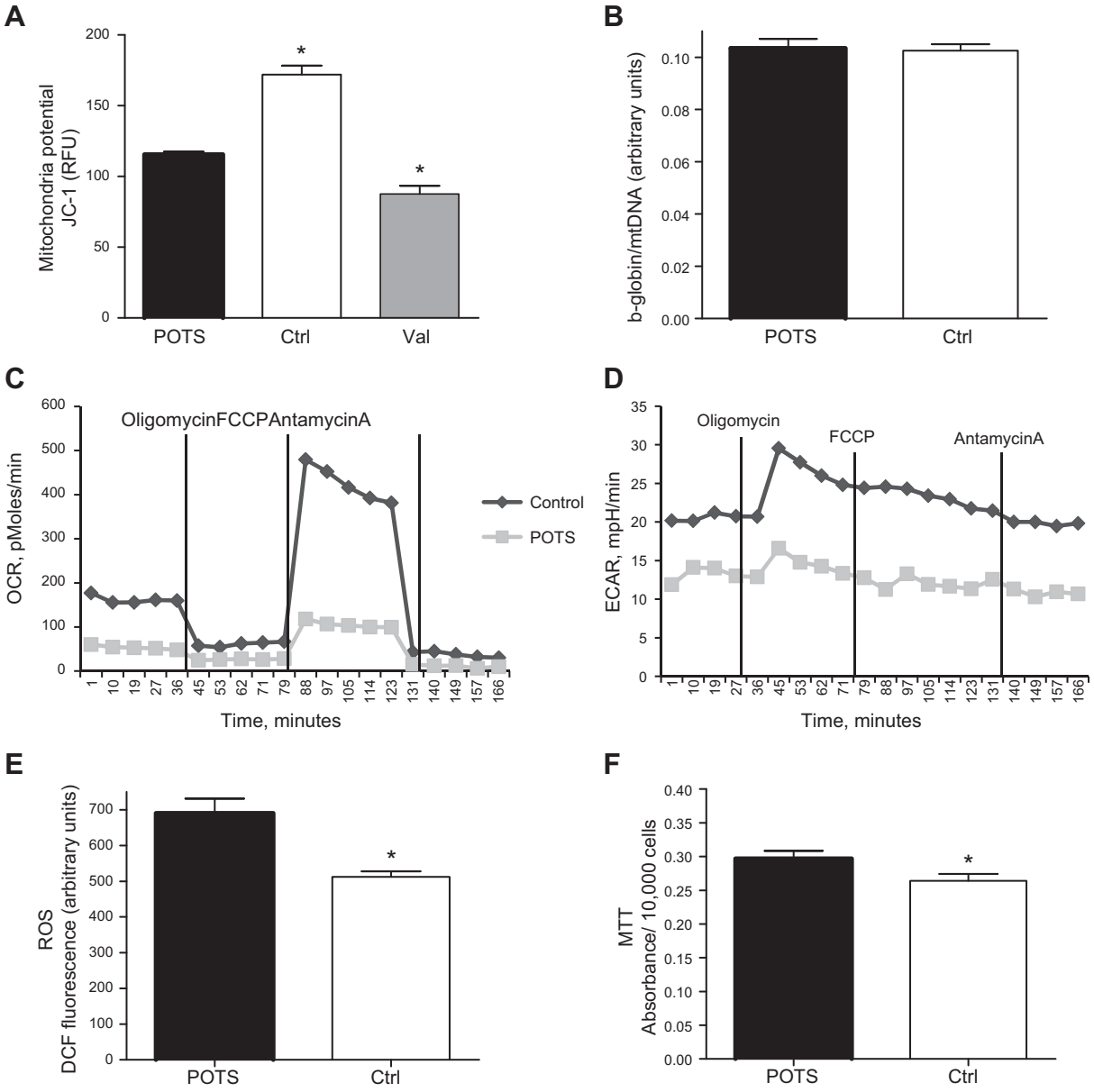

Figure 7. Mitochondrial capacity is reduced in POTS cells. *A*) Mitochondrial potential was reduced in POTS cells compared with control. Potential was measured using JC-1 staining, and fluorescence was read at 590 nm. Valinomycin (Val; 1 μg/ml) was used as an uncoupler agent and negative control. The result is expressed as relative fluorescence units (RFU). *$P < 0.01$ compared with POTS. *B*) mtDNA copy number relative to nuclear DNA content showed no difference in mitochondria number between POTS and control fibroblasts. JC-1 and mtDNA copy number were measured in triplicates. *C*) OCR and (*D*) ECAR results were lower in POTS than in control fibroblasts. *E*) ROS production, measured using the fluorescent probe DCF, was higher in POTS vs. control fibroblasts. *F*) Mitochondrial dehydrogenase activity, measured by MTT assay, was higher in POTS cells than in control fibroblasts. *$P < 005$ compared to POTS. OCR and ECAR and MTT assays were performed in 10 independent experiments, and ROS assay was performed in 6 replicates.

manner. CTL1/SLC44A1 could sense the substrate choline levels, and its function and expression were upregulated by choline supplementation. Therefore, choline treatment of POTS cells can increase the intracellular choline availability and can be an important component of the patent's therapy.

Our group has identified CTL1/SLC44A1-mediated choline transport in mitochondria of different cell types as a mode to control the pools of intracellular choline (14). CTL1/SLC44A1 is a choline/H^+ exchanger driven by the membrane electrochemical gradient (37), and the mitochondrial choline transport is dependent on the membrane potential (36). Choline deficiency in muscle cells reduces the amount of mitochondrial CTL1/SLC44A1 (7), and in the animal models, reduced choline decreases the mitochondrial membrane potential (38, 39). Here, we found that the mitochondrial choline uptake and mitochondrial potential are reduced in POTS cells. The impaired mitochondrial function was also present in POTS cells, confirmed by the reduced oxygen consumption (reduced respiration), glycolytic activity (reduced acidity), as well as an increased production of ROS. The

mitochondrial impairments in the POTS cells could be caused by dysfunctional membranes and modified phospholipid ratio and composition. Indeed, the composition of the mitochondrial membranes was previously found to be altered in choline deficiency, affecting mitochondrial oxidative capacity and energy metabolism (6).

The composition of membrane lipids is determined by species selectivity of the biosynthesis routes, fatty acid side-chain remodeling, and overall membrane turnover (3, 35, 40–42). Impaired choline transport is directly linked to altered PC, PE, and TAG metabolism in choline-deficient cells (7). We demonstrated that POTS cells have modified phospholipidomic profiles, reduced content of PC relative to PE and SM relative to FC, establishing a compromised membrane function in those cells.

The cellular characteristics of the POTS fibroblasts described here represent the first cell model of CTL1/SLC44A1 deficiency in which choline transport, general lipid homeostasis, and mitochondrial function were impaired. Choline treatment of the POTS fibroblasts could restore CTL1/SLC44A1 expression and choline uptake and offer a promising option for treating this patient. Because this is the first POTS study, it cannot be applied to other POTS cases. Whether POTS generally consists of choline and betaine deficiency and whether it has reduced choline transport and membrane function need to be firmly established in the future. The choline deficiency in this patient may be a result of an impaired supply of dietary choline by intestinal CTL1/SLC44A1 transporter (10, 43). However, further experiments on intestinal cell choline metabolism are needed to confirm this hypothesis. FJ

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