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Propionyl-CoA carboxylase *pcca-1* and *pccb-1* gene deletions in *Caenorhabditis elegans* globally impair mitochondrial energy metabolism

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Abstract

Propionic acidemia (PA) is a classical inborn error of metabolism with high morbidity that results from the inability of the propionyl-CoA carboxylase (PCC) enzyme to convert propionyl-CoA to methylmalonyl-CoA. PA is inherited in an autosomal recessive fashion due to functional loss of both alleles of either *PCCA* or *PCCB*. These genes are highly conserved across evolutionarily diverse species and share extensive similarity with *pcca-1* and *pccb-1* in the nematode, *Caenorhabditis elegans*. Here, we report the global metabolic effects of deletion in a single gene,

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either *pcca-1* or *pccb-1*, in *C. elegans*. Animal lifespan was significantly reduced relative to wild-type worms in both mutant strains, although to a greater degree in *pcca-1*. Mitochondrial oxidative phosphorylation (OXPHOS) capacity and efficiency as determined by direct polarography of isolated mitochondria was also significantly reduced in both mutant strains. While *in vivo* quantitation of mitochondrial physiology was normal in *pccb-1* mutants, *pcca-1* deletion mutants had significantly increased mitochondrial matrix oxidant burden as well as significantly decreased mitochondrial membrane potential and mitochondrial content. Whole worm steady-state free amino acid profiling by UPLC revealed reduced levels in both mutant strains of the glutathione precursor cysteine, possibly suggestive of increased oxidative stress. Intermediary metabolic flux analysis by GC/MS with 1,6-¹³C₂-glucose further showed both PCC deletion strains had decreased accumulation of a distal tricarboxylic acid (TCA) cycle metabolic intermediate (+1 malate), isotopic enrichment in a proximal TCA cycle intermediate (+1 citrate), and increased +1 lactate accumulation. GC/MS analysis further revealed accumulation in the PCC mutants of a small amount of 2-hydroxypropionate, which appeared to be metabolized in *C. elegans* to oxalate through a unique metabolic pathway. Collectively, these detailed metabolic investigations in translational PA model animals with genetic-based PCC deficiency have significantly dysregulated energy metabolism at multiple levels including reduced mitochondrial OXPHOS capacity, increased oxidative stress, and inhibition of distal TCA cycle flux, culminating in reduced animal lifespan. These findings demonstrate that the pathophysiology of PA extends well beyond what has classically been understood as a single PCC enzyme deficiency with toxic precursor accumulation, and suggest that therapeutically targeting the globally disrupted energy metabolism may offer novel treatment opportunities for PA.

Keywords

C. elegans; propionic acidemia; propionic aciduria; mitochondria; oxidative phosphorylation; TCA cycle

INTRODUCTION

Propionic acidemia (PA, OMIM #606054) is an inherited Mendelian disorder of intermediary metabolism having diverse clinical manifestations that range from intermittent metabolic acidosis induced by catabolic stress to life-threatening complications such as cardiomyopathy, metabolic stroke, and extrapyramidal neurological symptoms (Fenton et al 2001; Sutton et al 2012; Grunert et al 2013; Baumgartner et al 2014). PA has long been known to result from inactivating mutations in both alleles of either *PCCA* or *PCCB*, the two genes that encode subunits of the biotin-dependent propionyl-CoA carboxylase (PCC, EC 6.4.1.3) holoenzyme. PCC converts propionyl-CoA to methylmalonyl-CoA during the catabolism of methionine, threonine, isoleucine, valine, and odd chain fatty acids (Fenton et al 2001). Accumulation of propionic acid (and its related compounds) in the setting of PCC deficiency is commonly thought to be neurotoxic and the major metabolite responsible for the clinical disease sequelae of PA (DiDonato 2009). Illustrating known insights into the broader disruption of intracellular biochemical homeostasis that occurs, PA can also cause secondary urea cycle dysfunction due to the inhibitory effect of propionyl-CoA on carbonyl phosphate synthase (CPS1) with resulting hyperammonemia, (Grunert et al 2013).

Interestingly, many of the long-term clinical sequelae seen in PA patients resemble features seen in primary mitochondrial respiratory chain (RC) dysfunction (Haas et al 2007). Secondary mitochondrial dysfunction in PA has been postulated to result from accumulation of the endogenous toxin propionic acid and its downstream metabolites, 3-hydroxypropionate and methylcitrate (Schwab et al 2006). Indeed, prior studies have demonstrated that mitochondrial structure is disrupted in explanted organs obtained from PA patients (Schwab et al 2006; de Keyzer et al 2009) and in PA animal models (Gallego-Villar et al 2016).

Defining the precise metabolic alterations that contribute to the pathophysiology of PA is challenging in human patients. Model animals of metabolic disease offer an opportunity to address this challenge, allowing detailed dissection of complex biochemical phenotypes in controlled genetic and environmental backgrounds. *Caenorhabditis elegans* is a hermaphroditic nematode that has proven to be a robust *in vivo* animal model in which to examine the systemwide effects of primary biochemical disorders (Murfitt et al 1976; Wadsworth and Riddle 1989; Falk et al 2008; Rea et al 2010; Schrier et al 2014). *C. elegans* strains are readily accessible in a public repository for both classical missense mutations and transgenic knock-out alleles of many biochemical enzymes having known human disease equivalents (O’Riordan and Burnell 1989). Classical metabolic disorders modeled in diverse *C. elegans* models of primary mitochondrial RC deficiency have revealed identifiable biochemical signatures identifiable at the level of intermediary metabolite steady state levels and flux analyses, with characteristic disruption of tricarboxylic acid (TCA) cycle flux relative to wild-type animals (Falk et al 2008; Schrier et al 2014). Relevant to PA, the catabolism of branched chain amino acids, methionine, and threonine is highly conserved between humans and *C. elegans*, where only aspects of their synthetic pathways differ (http://www.genome.jp/kegg-bin/show_pathway?org_name=cel&mapno=00280&mapscale=1.0&show_description=show; http://www.genome.jp/kegg-bin/show_pathway?org_name=cel&mapno=00270&mapscale=&show_description=show; http://www.genome.jp/kegg-bin/show_pathway?org_name=cel&mapno=00260&mapscale=&show_description=show http://www.genome.jp/kegg-bin/show_pathway?org_name=cel&mapno=00260&mapscale=&show_description=show). Human *PCCA* and *PCCB* have close *C. elegans* orthologs, *pcca-1* and *pccb-1*, which respectively show 94.6% and 95.3% protein similarity by length (www.wormbase.org). Deletion of either *pcca-1* or *pccb-1* in *C. elegans* has been shown to not effect larval development (Chandler et al 2006), although adult lifespan effects have not been reported. Here, we examine the global metabolic alterations that occur in both *pcca-1* and *pccb-1* *C. elegans* knock-out gene models of PA, focusing on both *in vivo* and *in vitro* assays of integrated mitochondrial metabolism to better characterize the nature and extent of disrupted integrated RC capacity, TCA cycle flux, and mitochondrial content that occurs in PA.

MATERIAL AND METHODS

C. elegans strain growth and maintenance

The *C. elegans* strains N2 Bristol (wildtype), RB1774 (1754 basepair (bp) homozygous deletion of *pcca-1* (ok2282 allele)) and VC1307 (1301 bp homozygous deletion of *pccb-1* (ok1686 allele)) were studied (www.wormbase.org). All strains were obtained from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota, MN, USA. Nematodes were grown at 20°C on nematode growth media (NGM) plates spread with OP50 *Escherichia coli*. Synchronous young adults were obtained by bleaching gravid adults on NGM plates (Hope 1999), plating recovered eggs onto NGM plates without bacteria overnight, and then transferring L1-arrested nematodes to NGM plates spread with OP50 *E. coli*. Upon reaching adulthood (defined by visualization of eggs laid on plates), nematodes were washed off plates with S. basal (3.4 g KH₂PO₄, 4.4 g K₂HPO₄, 5.8 g NaCl, 1 liter water, pH.7.0). Adults were separated from eggs by gravity. All institutional and national guidelines for the care and use of laboratory animals were followed.

C. elegans lifespan analyses

Nematode lifespan analyses were performed, as previously described (Dingley et al 2010). Briefly, *C. elegans* cultures were synchronized by bleaching gravid adults and the next day transferring L1-arrested larvae to 10 cm NGM plates spread with OP50 *E. coli*. Upon reaching the first day of egg laying, synchronous young adults were moved to fresh 3.5 cm NGM plates seeded with OP50 *E. coli* (lifespan experiment 'Day 0'). Fluorodeoxyuridine (FUDR, 400 µM final concentration) was added to OP50-spread NGM plates to prevent eggs of these *C. elegans* adults from developing to reach adulthood (Hamilton et al 2005). The lifespans of both mutant strains were also repeated in the absence of FUDR to confirm alterations in the mutant strains' lifespans were not due to FUDR. 100 and 60 *C. elegans* worms of each *pcca-1* and *pccb-1* knockout strain and wild-type worms were studied, respectively, in each FUDR versus non-FUDR lifespan replicate experiment. Mortality was confirmed by stimulating nematodes lightly with a platinum wire; nematodes that did not move after stimulation were scored as dead and removed from the plate. Worms that died of protruding or bursting vulva, bagging, or crawling off the agar were censored. All lifespan studies were performed by a single technician (J.O.). Statistical analyses were determined in GraphPad Prism 5.0 for *pcca-1* and *pccb-1* knockout strains relative to concurrently studied wild-type worms.

In vivo mitochondrial fluorescence analyses in *C. elegans*

Terminal pharyngeal bulb microscopy analyses were performed to quantify mitochondria-localized fluorescence dye relative intensity changes between *C. elegans* mutant strains, as previously described (Dingley et al 2010). All studies were performed in the dark at 20°C. Briefly, synchronous young adult nematodes were incubated for 24 hours on NGM plates spread with OP50 *E. coli* and either 10 µM MitoSOX Red (Molecular Probes, Eugene, OR) to determine *in vivo* relative matrix oxidant burden (defined as the balance of mitochondrial matrix oxidant production and oxidant scavenging), 0.1 µM tetramethylrhodamine ethyl ester perchlorate (TMRE) (Invitrogen, Carlsbad, CA) to assess relative mitochondrial membrane potential, or 10 µM MitoTracker Green FM (Invitrogen, Carlsbad, CA) to

quantify relative mitochondrial content. After 24 hour incubation of animals with each fluorescent dye at 20°C, nematodes were collected and placed onto NGM plates spread with OP50 *E. coli* without any fluorescent dye for 1 hour to clear residual dye from their gastrointestinal tract. Living *C. elegans* worms were paralyzed directly on NGM plates using 1 mg/mL levamisole (Sigma, St. Louis, MO). Photographs were taken immediately in a dark room at 160X magnification and exposed for 2 seconds for MitoSOX Red, or 320 milliseconds for the other dyes, using a CY3 fluorescent cube set (MZFLIII, Leica, Bannockburn, IL) and a Cool Snap cf2 camera (Nikon, Melville, NY). Mean terminal pharyngeal bulb fluorescence was determined in approximately 50 nematodes per replicate, with three biological replicate experiments performed for each strain and fluorescent dye combination using NIS Elements Imaging Software (Nikon, Melville, NY), as previously described (Dingley et al 2010). Mean results from each fluorescence dye were compared for each worm strain and normalized to relative fluorescence levels for each mutant compared to levels in wild-type N2 Bristol control worms

Polarographic analysis of integrated respiratory chain capacity in freshly isolated mitochondria

Polarographic analysis in mitochondria isolated from synchronous adult worm populations was performed, as previously described (Falk et al 2006). Briefly, *pcca-1* and *pccb-1* knockout worm strains were grown in liquid culture. Freshly washed, synchronized, first day young adult worm populations were subjected to mitochondrial isolation procedures by differential centrifugation and homogenization, as previously described (Falk et al 2006). Polarographic analysis of freshly isolated mitochondria was performed using a Clark-type electrode (Oxytherm, Hansatech Instruments, United Kingdom), according to established protocol (Kayser et al 2003). All polarographic analyses were performed in technical duplicates with two biological replicate experiments per strain.

In vivo* stable isotope profiling of intermediary metabolic flux in *C. elegans

Stable isotope analyses were performed, as previously described (Falk et al 2011). Briefly, 2,000 nematodes of each strain were fed OP50 *E. coli* on NGM plates containing 5 mM [1,6-¹³C₂]-glucose from the L1-arrested stage throughout their approximately 3-day developmental cycle to assure sufficient isotope incorporation into all of their cells. Two biological replicate experiments were performed. Upon reaching young adulthood, nematodes were washed 3 times in 50 mL of S. basal buffer to remove residual bacteria. All reactions were then stopped by addition of perchloric acid to a final concentration of 4%. Nematodes were ground using a plastic homogenizer (Kontes Pellet Pestle) and motorized drill (Kontes Pellet Pestle Cordless Motor) for 15 seconds to disrupt their cuticle and cellular membranes. Precipitated protein was removed and redissolved in 1N NaOH, and quantified by DC Lowry Protein Assay (BioRad, Hercules, CA). Samples were returned to pH 7 with KOH (10 M). Organic acids were isolated using an AG-1 column (BioRad, Hercules, CA), and then derivatized for analysis of isotopic enrichment in by gas chromatography – mass spectrometry analysis (GC-MS, Metabolomics Core Facility, Children's Hospital of Philadelphia). Absolute levels and isotopic enrichment in molecular species of organic acids were analyzed in each mutant relative to wild-type animals, as previously described (Falk et al 2011).

Whole worm free amino acid level quantitation by UPLC in *C. elegans*

Whole worm free amino acid profiling was assessed by Ultraperformance® Liquid Chromatography (UPLC, Waters Aquity System) in the Metabolomics Core Facility at the Children's Hospital of Philadelphia Research Institute, as previously described (Narayan et al 2011). In brief, populations of 2,000 early larval stage (L1) worms were grown on NGM plates spread with OP50 *E. coli* until reaching the first day young adult stage, and then processed as detailed above for stable isotope studies until the stage of sample neutralization, except without isotope exposure and using SSA instead of PCA to stop the reaction. Amino acids were isolated from one aliquot of neutral sample using an AG-50W column (BioRad, Hercules, CA). Amino acids were derivatized in batches with a proprietary reagent, AccQTag® (Waters), which reacts with primary and secondary amines. Quantitation of whole worm amino acids was performed by direct injection of prepared samples by UPLC. At least three biologic replicate analyses were performed per strain/treatment.

Statistical Analysis

Standard descriptive statistics were calculated using Excel (Microsoft). Two-sample t-test was performed to assess the difference between two strains in the mean of the relative fluorescence intensity, the mean of the integrated oxidative phosphorylation (OXPHOS) rates, the mean of amino acid concentrations, and the mean of isotopic enrichment. Stable isotopic enrichment was calculated using Excel (Microsoft) for each experimental series according to the following formula: Atom Percent Excess, correct (APE) = $(R_{sa}-R_{st}) \times 100 / [(P_{sa}-P_{st}) + 100]$, where the R_{sa} is ratio of the sample and R_{st} is the ratio of the standard (Falk et al 2011).

RESULTS

C. elegans pcca-1 and *pccb-1* deletion mutants had shortened lifespans compared to wild-type worms

Lifespan was significantly reduced in both *pcca-1* and *pccb-1* deletion mutants relative to concurrently evaluated wild-type worms, both with (Figs 1A and S1A) and without (Figs 1B and S1B) the use of FUDR to prevent progeny development. Survival of both mutants was similar at younger ages but began to diverge by 8 to 9 days of adult life. Median lifespan was significantly reduced in both experiments in *pcca-1* (by 21–25% with FUDR and by 21–40% without FUDR relative to N2, $p < 0.0001$ in all conditions, Table S1). Median lifespan was also significantly reduced although to a more modest and variable extent in *pccb-1* relative to N2 (0–12% with FUDR and by 0–13% without FUDR, $p < 0.05$ in all conditions (Table S1). Thus, PCC deficiency due to either *pcca-1* or *pccb-1* deletion significantly reduced overall animal health, as assessed by the integrated physiologic measure of animal lifespan.

Deficiency of *pcca-1*, but not *pccb-1*, significantly reduced mitochondrial mass and mitochondrial membrane potential with induction of mitochondrial oxidative stress

Quantitation of terminal pharyngeal bulb mean relative fluorescence intensity following young adult nematode exposure for 24 hours to MitoTracker Green FM (mitochondrial

content), TMRE (mitochondrial membrane potential), and MitoSOX (mitochondrial matrix superoxide burden) was performed in living *pcca-1*, *pccb-1*, and N2 wild-type strains. Compared to wild-type nematodes, the *pcca-1* deletion strain had significantly reduced mitochondrial content (MTG) and mitochondrial membrane potential (TMRE, $p = 6.8 \times 10^{-6}$ and $p = 1.3 \times 10^{-6}$, respectively), with significantly increased mitochondrial matrix oxidant burden (MitoSOX, $p = 6.6 \times 10^{-25}$) (Figs 2 and S2). Interestingly, no significant variation in any of these mitochondrial parameters was seen in *pccb-1* deletion mutants relative to wild-type ($p > 0.05$ for all three assays) (Fig 2 and S2). *In vivo* mitochondrial mass and membrane potential of the *pcca-1* and *pccb-1* mutant worms significantly differed from one another ($p = 6 \times 10^{-4}$ and $p = 4.7 \times 10^{-3}$, respectively). The degree of reduced mitochondrial membrane potential seen in *pcca-1* was in excess of what could be attributed solely to the magnitude of its relatively reduced mitochondrial content. *pcca-1* mutants also had significantly greater *in vivo* matrix oxidant burden relative to *pccb-1* mutants ($p = 8.1 \times 10^{-24}$). Thus, *pcca-1* deficiency uniquely alters mitochondrial physiology, with mildly reduced mitochondrial mass, moderately reduced mitochondrial membrane potential, and substantially increased mitochondrial matrix oxidant burden.

Polarographic analysis of mitochondrial respiratory capacity in *pcca-1* and *pccb-1* mutants

To examine the direct impact of PCC deficiency on mitochondrial RC function in both *C. elegans* deletion strains, integrated oxidative phosphorylation (OXPHOS) capacity was measured by polarography in freshly isolated mitochondria (Figure 3 and Table S3). Multiple OXPHOS substrates were studied to test distinct aspects of integrated RC function. While no significant differences were seen between the *pcca-1* or *pccb-1* mutants and wild-type animals in background (no substrate) rate, both mutant strains had significant reductions in nearly all other oxygen consumption rates when tested with substrates indicative of complex I-dependent (malate), complex II-dependent (succinate), and complex IV-dependent (TMPD plus ascorbate) oxidative capacity (Figure 3). Specifically, state 3 (near-maximal) rates were significantly reduced by 37–45% for all substrates tested in *pcca-1* mutants and by 56–63% for all substrates tested in *pccb-1* mutants' mitochondria relative to wild-type ($p < 0.05$). This degree of reduction was suggestive of globally impaired mitochondrial respiratory capacity in both PCC mutant strains. No significant differences were seen in the respiratory control ratio (RCR) of either strain relative to wild-type for any substrate (Table S2), suggesting that both strains had normal coupling of oxidation to phosphorylation. However, complex I-dependent (malate) ADP/O ratios were significantly reduced in both strains ($p < 0.01$) and complex II-dependent (succinate) ADP/O ratio was significantly reduced in *pccb-1* mutant mitochondria ($p < 0.01$, Table S2), suggesting a reduced efficiency of ATP generation occurs in *pcca-1* with complex I substrates and in *pccb-1* with both complex I and II substrates. Overall, these data demonstrate that significant impairment of mitochondrial OXPHOS capacity occurs in both *pcca-1* and *pccb-1* models of PA.

Free amino acids were globally reduced in *pcca-1* and *pccb-1* deletion worm populations

Whole worm population free amino acid quantification was performed by UPLC to compare steady-state levels in both of the nematode models of PA to wild-type worms. The abundance of individual amino acids differs in *C. elegans* homogenized worm populations

from what is typical of human plasma, including lower levels of citrulline, glutamine, and ornithine amino acids involved in the ammonia processing system that is not well-conserved in *C. elegans*, higher levels of arginine which is the most predominant amino acid in *C. elegans* (Falk et al 2008), mildly increased levels of methionine and serine compared to human plasma. Overall, no significant changes were found in the levels of most amino acids in either mutant strain, with the exception of cysteine that was significantly reduced by over 2-fold in both *pcca-1* and *pccb-1* relative to N2 ($p < 0.05$) (Fig 4). Since cysteine is a precursor for the biosynthesis of a major antioxidant defense enzyme, glutathione, we postulate this finding may be indicative of increased oxidative stress in the PCC mutant strains, as was also suggested by *in vivo* oxidant burden analysis (Fig 2). Interestingly, no significant change was observed in either mutant in the level of alanine, which may be elevated in the setting of RC dysfunction (Clarke et al 2013; Schrier et al 2014). Whereas plasma glycine is typically increased in human PA patients (Lehnert et al 1994), glycine was not consistently elevated in the *pcca-1* and *pccb-1* deletion mutants compared to wild-type, possibly suggesting less inhibition of the glycine cleavage enzyme in worms than in humans from accumulation of propionyl-CoA and propionic acid. Overall, whole worm free amino acid profiling revealed reduced cysteine levels that may be indicative of increased oxidative stress, but no substantial alteration of amino acid metabolism in either *C. elegans* deletion model of PCC deficiency.

***pcca-1* and *pccb-1* *C. elegans* deletion strains have impaired distal TCA cycle flux with elevated lactate production**

Isotopic enrichment following 1,6-¹³C₂-glucose incubation was traced by GC/MS analysis of free metabolites in synchronous adult worm populations to interrogate *in vivo* metabolic flux through glycolysis, pyruvate metabolism, and the TCA cycle (Falk et al 2011; Schrier et al 2014). In these studies, +1 species isotopic incorporation is most informative of the relative metabolic progression of the labeled carbon from glucose through these major biochemical pathways. *pcca-1* or *pccb-1* deletion mutants are predicted to have compromised production of methylmalonyl-CoA, and subsequently, succinyl-CoA, which becomes incorporated into the TCA cycle. While we were not technically able by GC/MS to quantify succinyl-CoA, quantitation could be achieved of both citrate and malate which, respectively, are located proximally and distally to the TCA cycle entry site of succinyl-CoA (Fig 5A). Should TCA cycle flux be unaffected by an absence of carbon flow from succinyl-CoA in *pcca-1* and *pccb-1* deletion mutants, then similar levels of ¹³C label incorporation as in wild-type animals would be expected in all TCA cycle intermediates. However, this proved not to be the case (Fig S3). Indeed, both *pcca-1* and *pccb-1* deletion mutants had a 2-fold increased incorporation of +1 species enrichment in citrate (20.3 ± 5.1 and 22.1 ± 3.4 % APE, respectively) relative to wild-type nematodes (9.8 ± 2.2 % APE) ($p < 0.05$, Table S3). Although citrate can be produced by a number of metabolic pathways (e.g., citrate synthase, pyruvate dehydrogenase, pyruvate carboxylase), these studies suggest that the source of the measured increase was predominantly derived from the isotope-labeled glucose fed to the animals. The observed citrate enrichment in the PCC deficient worms is not likely to have originated from other carbon pools given the trend toward decreased to no isotopic incorporation into malate +1 species present in both PCC deficient nematode strains (0 ± 0 and 0.3 ± 0.5 % APE compared to wild-type worms 1.2 ± 1.9 % APE) (Table S3). Thus,

these isotopic enrichment metabolic flux data showed increased label in +1 citrate with a trend toward decreased label in +1 malate in both *pcca-1* and *pccb-1* deletion worm strains. These data suggest that while carbon flux proceeds from the labeled glucose precursor through glycolysis and pyruvate metabolism, it does not effectively flow from citrate through the TCA cycle to generate malate (Fig 5A).

Interestingly, both mutant strains also had significantly elevated enrichment in +1 lactate relative to wild-type N2 worms, with *pcca-1* and *pccb-1* nematode deletion mutants having 7-fold ($p < 0.05$) and 4-fold ($p < .01$) respectively, increased isotopic enrichment in +1 lactate (Fig S3 and Table S3). This result is consistent with similar findings in human PA patients who may develop lactic acidemia (Fenton et al 2001; Wendel 2006), as well as the observed impairment in RC function in the PCC mutant worm strains (Fig 3). Overall, these metabolic flux profiling data demonstrate that the PCC deficient *C. elegans* have increased lactate production and impairment of distal TCA cycle flux (Fig 5A).

Novel alternative propionate excretion pathway to form oxalate occurs in PCC mutant *C. elegans*

Stable isotopic-based analysis in whole worm population free organic acid extracts by GC/MS revealed no obvious differences, including in 3-hydroxypropionate or methylcitrate, in either PCC mutant worm strain relative to wild-type controls. Therefore, we postulated that these presumably toxic intermediaries are either absent, or if present, at low concentrations that fall below the GC/MS detection limit, in the PCC deficient nematodes. Support for the latter possibility comes from the recently reported detection using alternative techniques in *C. elegans* of 3-hydroxypropionate (Watson et al 2016). Therefore, we performed manual reanalysis of chromatogram peaks to confirm that 3-hydroxypropionate is indeed present at very low levels in both PCC mutants (Fig S4), where the relative amount was compared to the highest detectable peak, glycerate. Further analysis of related intermediary metabolites in these data are suggestive that propionate in PCC mutant *C. elegans* is metabolized through a unique alternative pathway, which involves: propionate's serial conversion to 3-hydroxypropionate, glycerate, and then hydroxymalonate that is decarboxylated to form glycolic acid, which is finally then converted to oxalate (Fig 5B). Indeed, whereas 3-hydroxypropionate levels were only present in PCC mutant worms at 2.1% that of glycerate, oxalate levels were present at 46% that of glycerate. Given that oxalate is poorly extracted in the analytic procedure, it is possible that *in vivo* oxalate levels are even higher in the PCC mutant *C. elegans* strains. Thus, we have identified a novel propionate excretion pathway that leads to increased oxalate production in *C. elegans* models of PA.

DISCUSSION

Deficiency of the PCC enzyme causes classical propionic acidemia (PA), with postulated sequelae extending beyond its immediate catabolic pathway to secondarily impair mitochondrial function. Here, we report direct evidence in *C. elegans* strains with deleted *pcca-1* or *pccb-1* that global mitochondrial dysfunction does occur in primary PCC deficiency, commonly resulting in significantly shortened animal lifespan, reduced

mitochondrial oxidative phosphorylation capacity, significantly reduced whole worm cysteine levels, increased lactate production, and reduced distal TCA cycle flux.

Despite these overarching effects that were consistent in both PA worm models, differences were detected in some aspects of mitochondrial physiology between the two PCC deletion worm strains. Specifically, relative quantitation of *in vivo* mitochondrial physiology with fluorescence dye microscopy analyses showed that deficiency of *pcca-1*, but not *pccb-1*, significantly reduced mitochondrial content and mitochondrial membrane potential, and significantly increased mitochondrial matrix superoxide burden (Fig 2). Interestingly, available evidence of mitochondrial effects in human PA patients presents similar variability, with some having mitochondrial depletion and others having OXPHOS complex deficiencies (Chemelli et al 2000; Mardach et al 2005; Schwab et al 2006; de Keyser et al 2009). However, unlike in worms, genotype does not appear to be the only contributing factor to phenotypic differences between human PA patients. In humans, *PCCA* encodes the carboxylase active site of PCC (Huang et al 2010), with absence of *PCCA* leading to degradation of *PCCB* (Ohura et al 1989). Human *PCCB* stabilizes the enzyme (Huang et al 2010), such that loss of *PCCB* results in a *de facto* loss of *PCCA*. This potentially explains why in human PA, the specific gene mutated (*PCCA* versus *PCCB*) does not impact the clinical severity of the disease phenotype (Perez-Cerda et al 2000; Kraus et al 2012). While human *PCCA* requires *PCCB* for stability, the same interaction may not be essential in *C. elegans* (Muro et al 2001). If *pcca-1* can exist independently of *pccb-1*, some PCC function could potentially be maintained in the *pccb-1* knockout worms. We postulate that the decreased mitochondrial content with increased mitochondrial oxidant burden that we observed only in the *pcca-1* deletion mutant may result from loss of its carboxylase function, although future studies will be necessary for confirmation. Therefore, despite both PCC deletion mutants having clear evidence of impaired OXPHOS capacity and distal TCA cycle flux, these data suggest that the two PCC proteins may differentially impact other aspects of *C. elegans* metabolism.

Interestingly, the amino acid profiles of the PCC mutants that indirectly effect the TCA cycle at the level of succinyl-CoA differed from what we had prior observed in primary TCA cycle deficient *C. elegans* mutant worms for the SDHC subunit, *mev-1* (Schrier et al 2014). Specifically, *mev-1* mutants had reduced aspartate with increased arginine and branched chain amino acid (isoleucine, leucine, valine) levels relative to wild-type worms (Schrier et al 2014), which were not seen here in the PCC deficient strains. Further, GC/MS-based 1,6-¹³C₂-glucose incorporation analysis in the *mev-1* mutants showed they had globally reduced TCA cycle flux, with significantly reduced incorporation into +1 molecular species of glutamate, aspartate, and citrate. Thus, TCA cycle flux was more globally impaired in the *mev-1* SDHC mutant than was observed here with predominant reduction only in distal cycle flux in the PCC mutants.

It is interesting to consider the basis of reduced distal TCA cycle flux in the PCC mutants. We hypothesized that reduced substrate entry into the TCA cycle at the level of succinyl-CoA caused by PCC deficiency in *pcca-1* and *pccb-1* deletion mutants would decrease the amount of unlabeled carbon in the molecular species of TCA cycle metabolites, with a corresponding greater percentage of label incorporated from ¹³C-glucose that enters and

flows through the TCA cycle via glycolysis and the pyruvate dehydrogenase reaction. However, our experimental data in PCC mutant worms demonstrated increased enrichment occurs in +1 citrate (a proximal metabolite in the TCA cycle) with a trend toward decreased enrichment in +1 malate (a distal metabolite in the TCA cycle). A plausible explanation for this observation in both PCC mutants may be that there is an increase in accumulation of citrate due to relatively decreased succinyl-CoA entry into the TCA cycle, resulting in decreased malate generation and accumulation of proximal intermediates prior to the entry point of succinyl-CoA including citrate. Another possible explanation for the observed differences in isotopic enrichment within TCA cycle intermediates may relate to an inhibitory role of propionic acid in particular components of the TCA cycle. While we attempted to perform isotopic analysis to validate this possibility by feeding worms ^{13}C -propionate, the chemical proved toxic to *C. elegans* and no incorporation could be detected in any intermediary metabolites (data not shown). However, the nematodes did have increased +1 lactate accumulation, which has been hypothesized to be due to pyruvate dehydrogenase complex inhibition (Schwab et al 2006) but may also occur in the setting of impaired TCA cycle flux. Prior studies using stable isotope perfusion of rat muscle illustrated that a complex system of TCA cycle regulation exists that may further complicate interpretation of these data (Lee and Davis 1979). That study showed when using lactate and pyruvate as substrates, isotopic enrichment was present in all portions of the TCA cycle, but using acetate as a substrate resulted in isotopic incorporation predominantly in the proximal C5/6 compounds (citrate, isocitrate, alpha-ketoglutarate) with reduced levels in distal C4 compounds (succinyl-CoA, succinate, fumarate, malate, oxaloacetate) unless pyruvate was provided in the reaction. Thus, pyruvate dehydrogenase activity is likely necessary to activate the alpha-ketoglutarate dehydrogenase reaction in the TCA cycle. Propionyl-CoA and propionate inhibition of pyruvate dehydrogenase in the setting of PCC deficiency would be anticipated to decrease the generation of acetyl-CoA, thereby further destabilizing the system and preventing the adequate conversion of citrate to replenish oxaloacetate in the TCA cycle (Gregersen 1981; Patel et al 1983).

More recently an alternative pathway for propionate metabolism has been described in *C. elegans* which is B12-dependent and initiated by *acdH-1*, a general acyl-CoA dehydrogenase (Watson et al 2016). Losing both *acdH-1* and *pcca-1* is lethal, although the impact of *acdH-1* and *pccb-1* has not been reported (Watson et al 2016). Having an alternative propionate metabolism pathway may contribute to subunit-specific sequelae of mutations in *pcca-1* vs *pccb-1* (Watson et al 2013). We further interpreted our GC/MS data to identify an additional unique propionate excretion pathway in *C. elegans* that leads to oxalate accumulation in the PCC mutant worms (Fig 5B). Future studies will be necessary to further delineate the relative contribution of these alternative metabolic pathways under typical and stress conditions in *pcca-1* versus *pccb-1* mutant worms.

In conclusion, multiple levels of dysregulated energy metabolism are evident in the *pcca-1* and *pccb-1* deletion *C. elegans* models of PA. Loss of either *pcca-1* or *pccb-1* reduces nematode lifespan, impairs mitochondrial OXPHOS capacity, reduces cysteine levels that may possibly be an indicator of increased oxidative stress, increases +1 lactate accumulation, inhibits distal TCA cycle flux, and increases oxalate production from a novel alternative propionate metabolic pathway of propionate to oxalate. Interestingly, differences

were seen between the two mutant strains in their *in vivo* mitochondrial physiology, where only deletion of *pcca-1* that contains the carboxylase active site had reduced mitochondrial content and membrane potential along with increased mitochondrial matrix oxidant burden. We also recognize that *C. elegans* has lower levels of PA, methylcitrate, and 3-hydroxypropionate relative to what is seen in humans, which are likely relevant biochemical factors that may contribute to biochemical differences observed between the two systems. Overall, these data suggest that future investigation of mitochondrial effects of *PCCA* and *PCCB* deficiency is warranted, including evaluation for different metabolic sequelae that may occur in different subclasses of human PA disease, with potential therapeutic opportunities to target mitochondrial dysfunction in the setting of primary PCC deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary

Two *C. elegans* model animals of propionic acidemia with single-gene *pcca-1* or *pccb-1* deletions have reduced lifespan with significantly reduced mitochondrial energy metabolism and increased oxidative stress, reflecting the disease's broader pathophysiology beyond a single enzyme deficiency with toxic precursor accumulation.

Figure 1. Lifespan analyses of PCC deficient *C. elegans* strains

Representative lifespan curves of *pcca-1* and *pccb-1* deletion nematodes relative to concurrent wild-type controls are shown for A. FUDR -exposed (to inhibit larval growth) and B. non-FUDR exposed animals. Both mutant strains had significantly decreased mean, median, and maximal lifespan compared to wild-type worms ($p < 0.001$) at both conditions. $n=100$ per biological replicate with FUDR, and $n=60$ per biological replicate without FUDR. Additional details are provided in Table S1, and replicate lifespan experiment figures are shown in Figure S1.

Figure 2. Fluorescence-based quantitation of relative nematode mitochondrial content, mitochondrial membrane potential, and mitochondrial matrix oxidant burden in *pcca-1* and *pccb-1* deletion mutants relative to wildtype nematodes

Terminal pharyngeal bulb fluorescence intensity normalized mean relative to wild-type worms is shown. Synchronous young adult nematode populations were incubated on NGM plates for 24 hours with either MitoTracker Green FM (MTG), TMRE, or MitoSOX Red (MitoSOX), respectively, to evaluate their relative mitochondrial content, mitochondrial membrane potential, and mitochondrial matrix oxidant burden. All results were normalized to wild-type mean fluorescence, indicated as 100 percent (horizontal black line). *, $p < 0.05$ and **, $p < 0.01$. Raw fluorescence data in arbitrary light units is shown in Figure S2.

Figure 3. Polarographic analysis of integrated OXPHOS respiratory capacity in isolated mitochondria from *pcca-1*, *pccb-1*, and wild-type nematodes

Integrated respiratory rates are shown for (A) complex I-dependent OXPHOS using malate as a substrate, (B) complex II-dependent OXPHOS capacity using succinate as a substrate, and (C) complex IV-dependent OXPHOS capacity using TMPD+ascorbate as a substrate. Rates include no substrate (background), state 3 (near-maximal), state 4 (ADP deficient), high ADP (maximal), DNP (duronitrophenol, uncoupled), and TMPD/Asc (complex IV specific) rates. Bars represent mean and standard error. $n = 4-6$ measurements per condition across 2 biological replicate experiments. *. $p < 0.01$ relative to wild-type controls. Additional details are provided in Table S2.

Figure 4. Whole nematode free amino acid profiling in *pcca-1* and *pccb-1* deletion mutants

Free amino acid levels were determined by UPLC (μmol/L). Each amino acid is listed and its values in triplicate biological replicate experiments are shown for each nematode strain. Diamonds mark the values for wild-type (N2), triangles for *pcca-1* mutants (PCCA), and circles for *pccb-1* mutants (PCCB). Two-sample t-test was performed for each two-way comparison between N2, *pcca-1*, and *pccb-1*, with p values detailed below each amino acid. Amino acids studied included alpha-amino butyric acid (AABA), alpha amino adipic acid (AADA), alanine (Ala), anserine (Ans), arginine (Arg), asparagine (Asn), aspartate (Asp), citrulline (Cit), cysteine (Cys), ethanolamine (EA), glutamine (Gln), glutamate (Glu), glycine (Gly), histidine (His), hydroxyproline (Hypro), leucine (Leu), isoleucine (Ile), lysine (Lys), methionine (Met), ornithine (Orn) phosphoethanolamine (PEA), phenylalanine (Phe), proline (Pro), phosphoserine (Pser), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val).

Figure 5. Whole worm metabolic flux analyses in *pcca-1* and *pccb-1* deletion mutant. (A) Schematic diagram of intermediary metabolites involved in glycolysis, pyruvate metabolism, and the TCA cycle that are interrogated by 1,6- $^{13}\text{C}_2$ -glucose incubation in *C. elegans*

This diagram is consistent with only one turn through the TCA cycle. Key amino acids that interact within these pathways are also illustrated. LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; CS, citrate synthase; ACO2, aconitase; IDH2/3, isocitrate dehydrogenase 2/3; OGDHc, 2-oxoglutarate dehydrogenase complex; SS, succinate synthase; SDH, succinate dehydrogenase; FH, fumarate hydratase; MDH2, malate dehydrogenase; PCC, propionyl-CoA carboxylase. Full filled circles are the carbons which would have a ^{13}C label is from 1,6- $^{13}\text{C}_2$ glucose. If label can be in one of two locations, the circles are half filled for visual clarity. A second succinyl-CoA which is unlabeled is included to highlight lack of label on first pass from carbons from the propionate pathway. In addition, unlabeled amino acids are also included in the figure to remind that those pools will include additional amino acids that have not been labeled. **(B) Novel alternative**

propionate excretion pathway to form oxalate occurs in PCC mutant *C. elegans*.

Percentages indicate levels relative to glycerate, which is the most predominant peak on GC/MS analysis (Fig S4). Results were consistent in both PCC mutant strains.