Molecular Genetics and Metabolism 122 (2017) 43–50

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Treatment with antioxidants ameliorates oxidative damage in a mouse model of propionic acidemia

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ARTICLE INFO

Keywords:
Propionic acidemia
Antioxidants
MitoQ
Resveratrol

ABSTRACT

Oxidative stress contributes to the pathogenesis of propionic acidemia (PA), a life threatening disease caused by the deficiency of propionyl CoA-carboxylase, in the catabolic pathway of branched-chain amino acids, odd-number chain fatty acids and cholesterol. Patients develop multisystemic complications including seizures, extrapyramidal symptoms, basal ganglia deterioration, pancreatitis and cardiomyopathy. The accumulation of toxic metabolites results in mitochondrial dysfunction, increased reactive oxygen species and oxidative damage, all of which have been documented in patients' samples and in a hypomorphic mouse model. Here we set out to investigate whether treatment with a mitochondria-targeted antioxidant, MitoQ, or with the natural polyphenol resveratrol, which is reported to have antioxidant and mitochondrial activation properties, could ameliorate the altered redox status and its functional consequences in the PA mouse model. The results show that oral treatment with MitoQ or resveratrol decreases lipid peroxidation and the expression levels of DNA repair enzyme OGG1 in PA mouse liver, as well as inducing tissue-specific changes in the expression of antioxidant enzymes. Notably, treatment decreased the cardiac hypertrophy marker BNP that is found upregulated in the PA mouse heart. Overall, the results provide in vivo evidence to justify more in depth investigations of antioxidants as adjuvant therapy in PA.

1. Introduction

Mitochondria are intracellular dynamic organelles that play an essential role in mammalian cell energy metabolism. Most cell energy is obtained through mitochondrial metabolic pathways, especially the Krebs cycle and electron transport chain that is the main site for production of reactive oxygen species (ROS). Energy-demanding tissues are highly sensitive to oxidative stress due to their high rate of oxygen consumption, substantial iron and polyunsaturated lipid contents, and relatively low activity of their antioxidant defence and repair enzymes [1]. Consequently, energy metabolism impairment and oxidative stress are relevant processes that have been involved in the pathophysiology of many human pathologies, including neurodegenerative, cardiovascular and inherited metabolic diseases (IMDs) [2–7].

One of the IMDs in which a secondary mitochondrial dysfunction and associated oxidative stress has been described is the rare neurometabolic disease propionic acidemia (PA, MIM#606054). PA, the most frequent, life-threatening organic acidemia with an incidence of 1 in 100,000 inhabitants, is caused by mutations in either the *PCCA* or *PCCB* genes, encoding both subunits of the mitochondrial propionyl-CoA carboxylase (PCC) enzyme. PCC is responsible for the generation of *D*-methylmalonyl-CoA from propionyl-CoA, which derives from the catabolism of some amino acids (isoleucine, valine, threonine and methionine), odd-chain fatty acids, and cholesterol [8]. The accumulation of propionyl-CoA is considered to be the major toxic agent, and in addition, there is the accumulation of metabolites of alternative propionate oxidation. Together they cause hyperammonaemia via inhibition of *N*-acetylglutamate synthetase and OXPHOS deficiency by synergistic inhibition of pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase complex and complex III, as well as inhibition of succinyl-CoA synthetase [9]. Besides, mtDNA depletion and ultrastructural mitochondrial abnormalities have been described in

Abbreviations: BNP, brain natriuretic peptide; CAT, catalase; CYPD, cyclophylin D; GPX1, glutathione peroxidase; MDA, malondialdehyde; OGG1, 8-oxoguanine DNA glycosylase 1; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances

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http://dx.doi.org/10.1016/j.ymgme.2017.07.009
Received 23 May 2017; Received in revised form 24 July 2017; Accepted 24 July 2017
Available online 25 July 2017

patients' tissues [9–12]. Increased intracellular hydrogen peroxide (H_2O_2) levels, correlating with the activation of the JNK and p38 signalling pathways, and high levels of oxidative stress markers have also been observed in patient-derived fibroblasts and urinary samples [13,14]. Furthermore, we have shown that the hypomorphic PA mouse model (*Pcca*$^{-/-}$(A138T)) showed tissue-specific alterations in OXPHOS complexes and/or activities, mtDNA depletion, an increase in superoxide production and H_2O_2 levels, as well as variations in antioxidant defences and lipid oxidative damage [15].

Clinical presentation of PA varies from a severe, early-onset form of the disease, with neonatal ketoacidosis, lethargy, failure to thrive and encephalopathy, to a milder late-onset presentation usually diagnosed during infancy which has a less serious neurological outcome [12]. Long-term complications have also been described including growth retardation and developmental delay, poor cognitive outcome and progressive neurological dysfunction by basal ganglia degeneration, skin lesions, pancreatitis, cardiomyopathy and rhythm disturbances [12]. If not promptly and appropriately treated, patients can die or develop permanent brain damage. Conventional treatment is based on dietary restriction, carnitine and biotin supplementation, and metronidazole, and these have allowed patients to live beyond the neonatal period [16].

Despite improved therapy over the past few decades, the outcome of PA patients is still unsatisfactory, highlighting the requirement to evaluate new therapies. Considering the hypothesis that secondary mitochondrial dysfunction resulting in increased ROS and oxidative damage plays a major role in the pathomechanisms of PA, we have focused our research in the potential therapeutic applications of antioxidant compounds in this disease. In a previous study using patient-derived fibroblasts we observed that different antioxidants (Tiron, Trolox, resveratrol and MitoQ) significantly reduced H_2O_2 levels and also regulated the expression of antioxidant enzymes [17]. In this work we have used the hypomorphic murine model of PA to investigate the in vivo effects of two selected antioxidants acting on mitochondrial oxidative stress: i) mitochondrial targeted MitoQ, which is thought to act primarily by decreasing mitochondrial lipid peroxidation [18] and ii) resveratrol, with potent antioxidant properties acting through various mechanisms, including the upregulation of antioxidant enzymes and the enhancement of mitochondrial biogenesis [19].

2. Materials and methods

2.1. Mice handling

All mice used, wild-type and hypomorphic *Pcca*$^{-/-}$(A138T) (kindly provided by Prof. M.A. Barry, Mayo Clinic, US) [20], were adult males or females in an FVB background. Mice were maintained on standard chow. Animal experiments were carried out in a pathogen-free environment at the Animal Facility of Centro de Biología Molecular Severo Ochoa, in accordance with the Spanish Law on Animal Protection. All animal studies were approved by the Institutional Animal Experimentation Ethical Committee (Universidad Autónoma de Madrid, reference CEI 963-A026) and by the Regional Environment Department (Comunidad de Madrid, reference PROEX 22/14). Genotyping was performed using genomic DNA isolated from tail biopsies as previously described [20,21].

2.2. Antioxidant treatment trials

Wild-type and hypomorphic *Pcca*$^{-/-}$(A138T) mice were randomly divided into the following groups: non-treated [wild-type (n = 12) and *Pcca*$^{-/-}$(A138T) (n = 11)], MitoQ-treated [wild-type (n = 12) and *Pcca*$^{-/-}$(A138T) (n = 11)] and resveratrol-treated [wild-type (n = 10) and *Pcca*$^{-/-}$(A138T) (n = 11)]. Mice were 7 months of age at the start of both treatments. MitoQ was administered at a concentration of 250 μM in drinking water for 3 months. In a previous study, doses up to 500 μM showed no toxicity and had no significant effect on animal body weight or food and liquid consumption [22]. The resveratrol group received 30 mg/L of resveratrol (Sigma-Aldrich, St. Louis, MO, USA) in their drinking water for 2 months. Fresh MitoQ and resveratrol solutions were prepared twice a week and administered in light-protected water bottles. Water intake was monitored weekly until the end of the study.

2.3. Western blotting

Wild-type and hypomorphic *Pcca*$^{-/-}$(A138T) mice were euthanized and brain, heart and liver tissues were immediately excised, snap-frozen in liquid nitrogen and stored at −70 °C until processed. Proteins from mouse tissues were isolated by disrupting 50–100 mg wet weight of the mice organs in lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM DTT, 1% Triton X-100, 0.1% SDS, 0.4 mM EDTA) using a T10 basic Ultra-Turrax homogenizer (IKA, Germany) and centrifuged 30 min at 4 °C. The supernatant fraction was collected and protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of lysed extracts (75 μg protein) were loaded on a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane (iBlot® Gel Transfer Stacks, Regular) in an iBlot® Gel transfer device (Invitrogen, Carlsbad, CA, USA). Immunodetection was carried out using commercially available antibodies against MnSOD (1:1000; Enzo Life Sciences Cat# ADI-SOD-110, RRID:AB_10616816), GPx1 (1:1000; Abcam Cat# ab22604, RRID:AB_2112120), CAT (1:500–1:1000; Abcam Cat# ab22604, RRID:AB_2112120), CypD (1:1000; Abcam Cat# ab110324, RRID:AB_10864110) and OGG1 (1:1000; Novus Cat# NB100-106, RRID:AB_10104097). Secondary antibodies used were goat anti-rabbit (1:5000; Santa Cruz Biotechnology Cat# sc-2004, RRID:AB_631746) or goat anti-mouse (1:5000; Santa Cruz Biotechnology Cat# sc-2005, RRID:AB_631736). For loading control, membranes were immunostained with GAPDH antibody (1:5000; Abcam Cat# ab8245, RRID:AB_2107448). Antibody binding was detected by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK). Protein quantification was performed using a calibrated densitometer GS-800 (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. RNA isolation and quantitative real-time PCR

Total RNA was extracted from the heart using miRCURY™ RNA Isolation Kit-Tissue (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. RNA yield was assessed using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Rockland, DE, USA). 250 ng of total RNA was retrotranscribed using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisboa, Portugal). The *Nppb* gene (coding for the pro-hypertrophic marker BNP) was amplified with specific primers previously described [23] using PerfeCTa SYBR Green Fast Mix Kit (Quantabio, Beverly, MA, USA) in a LightCycler480 II (Roche Applied Biosciences, In, USA) instrument. GAPDH (Forward primer: AGCTGAACGGGAAGCTCACT; Reverse primer: GCTTCACCACCTTCTTGATGTC) was used as endogenous control. All samples were run in triplicate and mRNA relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5. TBARS assay

Thiobarbituric acid reactive substances (TBARS) were measured using Oxiselect TBARS MDA quantitation kit (Cell Biolabs, San Diego, CA, USA). 50 mg of frozen tissue were homogenized using a T10 basic Ultra-Turrax homogenizer (IKA, Germany) and malondialdehyde (MDA) was measured following the manufacturer's instructions. Each sample was run in triplicate using a FLUOstar OPTIMA (BMG LABTECH, Ortenberg, Germany) fluorescence plate reader. Tissue protein content was measured using the Bradford method (Bio-Rad