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Antioxidants successfully reduce ROS production in propionic acidemia fibroblasts

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ABSTRACT

Propionic acidemia (PA), caused by a deficiency of the mitochondrial biotin dependent enzyme propionyl-CoA carboxylase (PCC) is one of the most frequent organic acidurias in humans. Most PA patients present in the neonatal period with metabolic acidosis and hyperammonemia, developing different neurological symptoms, movement disorders and cardiac complications. There is strong evidence indicating that oxidative damage could be a pathogenic factor in neurodegenerative, mitochondrial and metabolic diseases. Recently, we identified an increase in ROS levels in PA patients-derived fibroblasts. Here, we analyze the capability of seven antioxidants to scavenge ROS production in PA patients' cells. Tiron, trolox, resveratrol and MitoQ significantly reduced ROS content in patients and controls' fibroblasts. In addition, changes in the expression of two antioxidant enzymes, superoxide dismutase and glutathione peroxidase, were observed in PA patients-derived fibroblasts after tiron and resveratrol treatment. Our results in PA cellular models establish the proof of concept of the potential of antioxidants as an adjuvant therapy for PA and pave the way for future assessment of antioxidant strategies in the murine model of PA.

1. Introduction

With an incidence of 1 in 150,000 inhabitants, propionic acidemia (PA; MIM#606054) is the most frequent organic acidemia. The two main phenotypes of PA are a severe form with neonatal ketoacidosis, feeding refusal, lethargy, failure to thrive, seizures and encephalopathy; and a milder, later-onset chronic form. The disease is caused by mutations in either the *PCCA* (MIM#23200) or *PCCB* (MIM#232050) genes encoding the α- and β-subunits of propionyl-CoA carboxylase protein (PCC, EC 6.4.1.3). PCC is a mitochondrial biotin-dependent enzyme that catalyzes the conversion of propionyl-CoA to D-methylmalonyl-CoA in the catabolic pathway of the essential amino acids threonine, methionine, valine and isoleucine, odd-chain fatty acids and cholesterol [1].

Major secondary biochemical abnormalities including hyperammonemia, hypoglycemia, hypocarnitinemia, lactic acidosis and an increased synthesis of odd-numbered long-chain fatty acids (OLCFA) are due to the intracellular accumulation of propionyl-CoA and other organic acids and esters [2]. In addition, inhibition of the TCA cycle enzyme succinyl-CoA synthetase, a significant decreased activity of respiratory chain complexes, mtDNA depletion and ultrastructural mitochondrial abnormalities have been described in patients' tissues, supporting the role of a secondary mitochondrial dysfunction in the development of the multiorgan complications observed [3–6].

We have recently described the presence of increased intracellular reactive oxygen species (ROS) content in PA patients-derived fibroblasts compared to controls, correlating with the activation of the JNK and p38 signaling pathways and suggesting the presence of oxidative damage in these cells which could be related to the pathophysiology of the disease [7]. In this context, urinary samples from eight PA patients have shown high levels of oxidative stress markers [8], and propionic acid was demonstrated *in vitro* to stimulate the production of superoxide anion in presence of Ca^{2+} influx activators in human neutrophils [9], to stimulate lipid peroxidation in rat cerebral tissues [10] and to increase protein carbonylation in rats [11]. Antioxidant treatment with ascorbic acid was able to prevent neurological deficits provoked by *in vivo* acute administration of propionic acid in rats suggesting that oxidative stress may be involved in the neuropathology of PA [12].

Progression of PA symptoms may lead to death within a few days or to severe brain damage if not promptly treated. Currently, several strategies exist to treat the disease, including the combina-

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tion of biotin and metronidazole, the restriction of precursor amino acids, the supplementation of L-carnitine and the prevention of the catabolic state. However, the overall outcome of PA patients remains disappointing, a variety of neurological abnormalities and cardiac, hematological and renal complications are frequent even under apparently good metabolic control [13], reflecting the poor knowledge of the underlying pathophysiological mechanisms. For this reason additional treatments for PA are needed. Considering these facts and the reported oxidative stress in PA patient samples, we set out to test different antioxidants to scavenge ROS production in PA patients-derived fibroblasts.

2. Materials and methods

2.1. Chemical reagents

Resveratrol, trolox (±6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), tiron ($C_6H_4Na_2O_8S_2$), N-acetyl-L-cysteine (NAC), melatonin and alfa-tocopherol (vitamin E), all of them were obtained from Sigma–Aldrich, St. Louis, MO. Triphenylphosphonium (TPP) and MitoQ were kindly provided by Dr. M. Murphy (Cambridge, UK).

2.2. Patients and controls

A total of 10 patients diagnosed with PA based on their clinical symptoms and on biochemical analysis were included in this study. The patients' molecular and available clinical data have been previously described [7]. Control fibroblasts were obtained from Lonza Inc., (Allendale, NJ, USA): CC2509 and CC2511. Ethical approval was obtained from the institutional Ethics Committee of the Universidad Autónoma de Madrid for the use of human samples in the present study.

2.3. Cell culture and treatments

Primary fibroblasts from PA patients were cultured in Minimum Essential Medium supplemented with 1% glutamine 200 mM, 10% fetal bovine serum and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL) in a humidified atmosphere containing 5% CO_2 at 37 °C. Assays were performed when the cell cultures reached 75–85% confluence. Control and patients-derived fibroblasts were used between passages 10 and 15.

Twenty-four hours after seeding cells were treated with antioxidants at different times and concentrations, according to published procedures: tiron (40 mM) for 18 h; resveratrol (1 mM) for 3 h; trolox (1 mM) for 72 h; TPP (vehicle) and MitoQ (200 nM) for 24 h; NAC (1–10 mM) for 1–4 h; vitamin E (30 μM) for 18 h; and melatonin (20–100 μM) for 24 h.

2.4. Determination of intracellular ROS levels

Hydrogen peroxide (H_2O_2) levels were monitored to determine intracellular ROS using 2′,7′-dichloro-dihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Inc., Eugene, OR) and flow cytometry as described [14].

2.5. Western blot analysis of MnSOD and GPX1 protein expression

Protein extracts were obtained as described [15]. Proteins (20 μg) were separated using 4–12% NuPAGE Novex Bis-Tris mini gels (Invitrogen, Carlsbad, CA, USA) and transferred to Whatman nitrocellulose membranes at 20 V for 7 min using iBlot system (Invitrogen). Protein equal loading was assessed using Poinceau S staining. Membranes were blocked for 1 h at 4 °C with Tris-buffered saline (TBS) with 0.05% Tween-20 and 5% non-fat milk. Blots were incubated with primaries antibodies according to assay: SOD-2 (1:10,000, Enzo Life Sciences, Lausen, Switzerland), Hsp60 (1:5000, Enzo Life Sciences) and GPX1 (1:1000, Abcam, Cambridge, UK). Secondary antibodies were used as IgG-horseradish peroxidase conjugated (anti-rabbit (1:5000) and goat anti-mouse (1:5000)) and were detected by Enhanced Chemiluminescence System (GE Healthcare, Berkshire, UK). Protein quantification was performed using a calibrated densitometer GS-800 (Bio-Rad Laboratories, Hercules, CA, USA).

3. Results and discussion

Our previous studies showed an increase of intracellular ROS and apoptosis content in PA fibroblasts [7]. A consequence of ROS production is their interaction with biomolecules (DNA, lipids and proteins) which are then modified and functionally altered. If oxidative damage plays a relevant role in PA pathophysiology, an ideal additional strategy for PA treatment would be the use of different antioxidant compounds acting through complementary mechanisms for ROS scavenging.

We investigated the potential of several agents to scavenge ROS production generated in fibroblasts from a total of eight *PCCA* deficient and two *PCCB* deficient patients. The compounds tested were vitamin E, trolox, tiron, NAC, melatonin, resveratrol and MitoQ. Vitamin E and its analog trolox were chosen as they can inhibit the propagation phase of the peroxidative process by neutralizing the lipid-derived radicals; tiron as it can scavenge superoxide anion; NAC as it can regenerate reduced glutathione and eliminate several ROS (OH^-, H_2O_2, peroxyl radicals and nitrogen-centered free radical); melatonin as it is a direct free radical scavenger, stimulates antioxidant enzymes and increases the efficiency of mitochondrial oxidative phosphorylation and other antioxidants; resveratrol as it can inhibit lipid peroxidation and can directly scavenge ROS; and MitoQ as it can prevent the disruption of mitochondrial function caused by oxidative stress.

Tiron, MitoQ, trolox and resveratrol were capable individually of decreasing ROS levels in all PA patients-derived fibroblasts and controls (Figs. 1 and 2). However NAC, melatonin and vitamin E displayed no effects in ROS content in cell samples (data not shown).

The use of tiron showed a significant decrease in ROS levels (50–80%) in all patients-derived fibroblasts (Fig. 1A). The largest decrease (about 80%) was in P1 which was one of the two PA patients' fibroblasts who previously presented the highest ROS levels [7]. There is also a group of patients (P3, P7, P8 and P9) who presented a decrease of 50% in ROS content and previously presented a moderate increase in ROS levels (~1.5-fold).

MitoQ and trolox scavengers displayed a moderate decrease in ROS levels (MitoQ: 25–50% and trolox: 15–30%) (Fig. 1B and C). Fibroblasts from P1 again showed the largest decrease in ROS levels (50% with MitoQ and 30% with trolox).

Intracellular ROS levels were decreased about 30–40% by using resveratrol in P1–P5 patients-derived fibroblasts (Fig. 2A). It is worth noting that resveratrol at 1 mM concentration (Fig. 2B) showed cellular cytotoxicity as has been previously described [16,17], which was not observed with the previously described antioxidants. Cytotoxic effects of resveratrol were not observed when used in retinal epithelial cells at a concentration of 25–100 μM [18]. We have also tested lower concentrations of resveratrol (25–500 μM) and a moderate decrease of ROS content (~20%) was observed at 200–500 μM (data not shown). Although resveratrol shows potential for exerting beneficial effects in reducing ROS levels in PA patients-derived fibroblasts, dosing should be carefully adjusted in future *in vivo* studies in murine models.

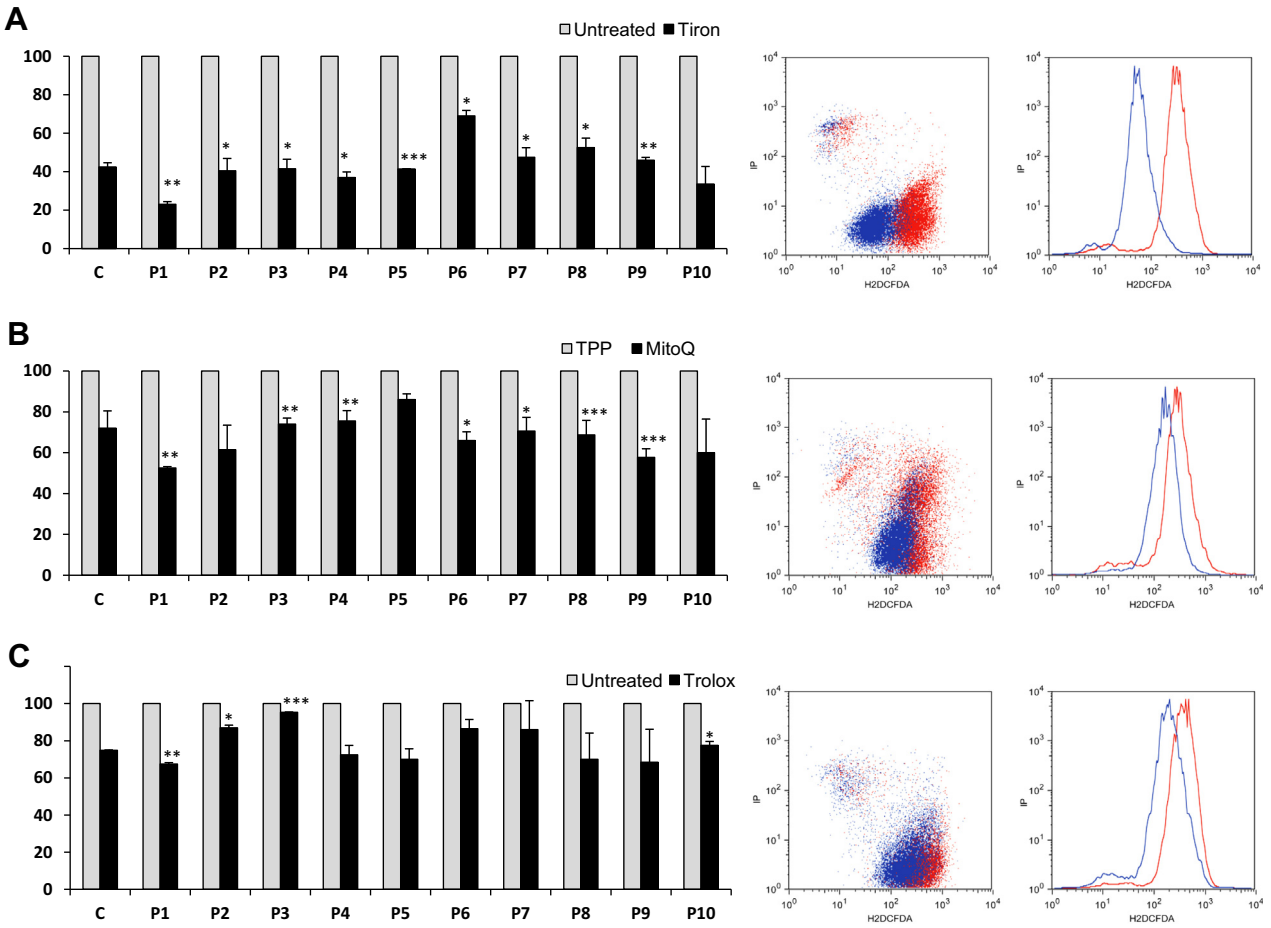

Fig. 1. Detection of ROS levels by flow cytometry in controls and PA patients-derived fibroblasts after antioxidant treatment. (A) Tiron treatment. Cells were incubated with 40 mM of tiron for 18 h. (B) MitoQ treatment. Fibroblasts were incubated with 200 nM of MitoQ for 24 h. (C) Trolox treatment. Cells were incubated with 1 mM of trolox for 72 h. Results are expressed as percentage of cells in antioxidant-treated fibroblasts relative to the corresponding untreated cells (tiron and trolox) and to the corresponding vehicle TPP treated cells (MitoQ). Data represent mean ± SD of three independent experiments, each one performed in triplicate ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). C: control; P: patient. Panels on the right are representative experiments of ROS levels in untreated cells (red) and antioxidant-treated fibroblasts (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Each of the antioxidants tested may contribute to the decrease in ROS levels through different mechanisms that may be operating in PCC deficient cells. We were able to reduce ROS production *in vitro* using tiron suggesting that the generation of superoxide contributes critically to the oxidative damage in the PA fibroblasts studied. Trolox (the polar analog of vitamin E) could aid in scavenging lipid peroxyl radicals as it is a well-known lipid antioxidant which forms complexes with free saturated and unsaturated fatty acids in cell membranes, therefore neutralizing the detergent-like properties of the hydrolytic products that would otherwise disrupt membrane stability [19]. Resveratrol is a polyphenolic phytoalexin found in red grapes and other plant products and its protective effects are mostly attributable to its effects on redox enzymes rather than its moderate direct ROS-scavenging activity [20].

In our work, MitoQ which is the most widely used mitochondria-targeted antioxidant to date, also scavenged ROS production significantly in PA patients-derived fibroblasts. MitoQ comprises a triphenylphosphonium (TPP) functionality conjugated to an antioxidant ubiquinone moiety [21]. MitoQ is accumulated within mitochondria *in vivo* in response to the large mitochondrial membrane potential (negative inside) and protects mitochondria from oxidative damage. MitoQ has been shown to mitigate oxidative damage in various disease animal models [22], including Alzheimer [23] and Parkinson disease [24].

Cells regulate ROS levels using enzymatic and nonenzymatic cellular defense mechanisms. Superoxide dismutase and glutathione peroxidase, two strong mitochondrial antioxidant enzymes, are the primary antioxidant defense components that catalyze the conversion of superoxide anion to H_2O_2 and the decomposition of H_2O_2 to water, respectively. The activity or presence of antioxidant enzymes in cells is strongly regulated by transcriptional, translational and post-translational mechanisms as a consequence of changes in the cellular redox status [25]. To analyze the correlation of oxidative stress with antioxidant enzymes' expression, we examined the expression of superoxide dismutase (MnSOD) and glutathione peroxidase (GPX1) in several of the PA patients-derived fibroblasts (selected based on the availability of cells with fewer cell passage number). Cells were analyzed in basal conditions or after the incubation with two ROS scavengers (Fig. 3). Resveratrol and tiron were selected as they resulted in the most significant decrease in ROS levels in PA cells. In untreated cells we could detect an alteration in antioxidant defenses in PA fibroblasts; GPX1 levels were decreased relative to the control cell line, while mitochondrial MnSOD levels were generally increased, in line with previous observations [7].

The expression of MnSOD and GPX1 after resveratrol treatment increased in the patients' cells used (Fig. 3). Resveratrol was recently reported to regulate mitochondrial gene expression, being a key factor in the regulation of cellular defenses and cell survival

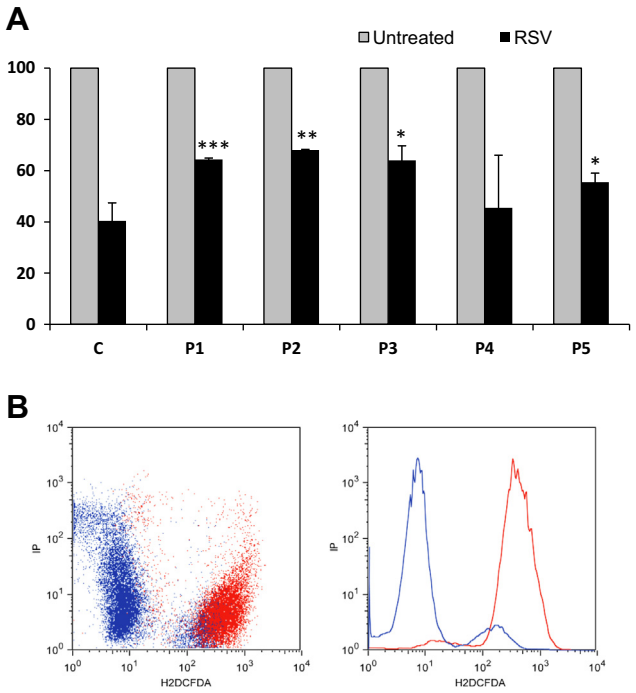

Fig. 2. Detection of ROS levels by flow cytometry in controls and PA patients-derived fibroblasts after resveratrol treatment. (A) Cells were incubated with 1 mM of resveratrol for 3 h. Results are expressed as percentage of cells in resveratrol-treated fibroblasts relative to the corresponding untreated cells. Data represent mean ± SD of three independent experiments, each one performed in triplicate (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). C: control; P: patient; RSV: resveratrol. (B) A representative experiment of ROS levels in untreated cells (red) and resveratrol-treated fibroblasts (blue) is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

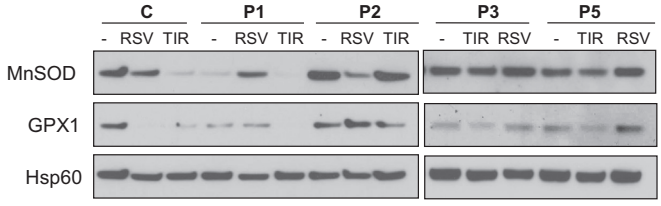

Fig. 3. Analysis of superoxide dismutase (MnSOD) and glutathione peroxidase (GPX1) expression in controls and PA patients-derived fibroblasts. Cells were incubated with 1 mM of resveratrol for 3 h and with 40 mM of tiron for 18 h. Equal amounts of protein from control and patients fibroblast samples (20 μg of total cell lysates) were loaded and subjected to Western blot analysis with anti-MnSOD and anti-GPX1 antibodies. The bottom panel shows the stripped blots reprobed with Hsp60 antibody to ensure equal amounts of protein loaded in each lane. This result is representative of three independent experiments. C: control; P: patient. RSV: resveratrol; TIR: tiron; -: cells not treated.

responses to stress [26]. This antioxidant induces protein expression of mitochondrial oxidation respiratory complexes, several transcription factors and SOD in cardiomyocytes [27]. Resveratrol upregulates MnSOD and GPX1 in human endothelial cells [28]. It is interesting to note that GPX activity is regulated by post-translational events, such as phosphorylation at specific tyrosine residues [29] and that resveratrol increasing GPX phosphorylation level, thereby enhancing its enzymatic activity [30]. In addition, since MnSOD converts superoxide to hydrogen peroxide, an increase in MnSOD expression in resveratrol-treated cells suggests that resveratrol exposures could alter cellular superoxide levels.

PA patients and controls-derived fibroblasts treated with tiron significantly decreased the expression of MnSOD and GPX1 proteins (Fig. 3). The O_2^- decrease produced by treatment of the nonenzymatic superoxide scavenger tiron could explain the decrease observed in MnSOD protein expression in PA fibroblasts. In addition, reduced antioxidant GPX1 enzyme activity was detected in coronary endothelial cells treated with tiron [31].

In summary, the results presented in this work indicate that several antioxidant agents effectively reduce ROS production in fibroblasts from patients with PA, providing for the first time experimental evidence of the feasibility of antioxidant therapy in this organic acidemia. Oxidative stress and damage are involved in a wide variety of neurodegenerative, mitochondrial and metabolic diseases having a secondary role in disease pathophysiology, and clinical trials using several antioxidants have demonstrated that these compounds are able to improve different symptoms associated with the disease [22]. Based on the results presented in this work, the next step will be the *in vivo* analysis of oxidative stress and potential beneficial effects of the selected antioxidants in the recently generated hypomorph murine model of PA [32]. This murine model survives up to adult age while mimicking the clinical and biochemical hallmarks of the disease thus constituting an adequate model for preclinical studies of antioxidant strategies as adjuvant therapy for PA.

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