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In Vitro Study on the Response of Fibroblast Cellular Respiration to Lipoic acid, Thiamine and Carnitine in Patients with Dihydrolipoyl Dehydrogenase Deficiency

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Abstract

Objectives: This study examined *in vitro* responses of fibroblast cellular respiration to lipoic acid, thiamine and carnitine in patients with dihydrolipoyl dehydrogenase (DLD) deficiency. This disorder impairs cellular bioenergetics and these compounds are used to improve clinical manifestations of the disease. The study aimed to utilize mitochondrial O_2 consumption as a surrogate biomarker for examining cellular responses to metabolic therapies.

Methods: Cultured fibroblasts from three patients were treated with therapeutic concentrations of the compounds for 24 hours. Cells were then harvested and processed for measuring respiration using phosphorescence oxygen analyzer. Patients 1 and 2 were severely symptomatic infants with homozygous c.1436A>T mutation in the DLD gene. Patient-3 was a mildly symptomatic adolescent with homozygous c.685G>T mutation.

Results: The rate of respiration (mean ± SD, n=6, μM O_2 $min^{-1}/10^7$ cells) in fibroblasts from a normal infant was 9.3 ± 1.6, in fibroblasts from Patient-1 was 5.1 ± 0.9 (p=0.001), in fibroblasts from Patient-2 was 7.4 ± 1.4 (p=0.051), and in fibroblasts from Patient-3 was 10.3 ± 3.3 (p=0.836). In normal fibroblasts, respiration decreased by the thiamine (p=0.012) and carnitine (p=0.023) treatments. In Patient-1, respiration increased by the lipoic acid (p<0.002), thiamine (p<0.001), and carnitine (p=0.018) treatments; this patient clinically responded to thiamine. In Patient-2, respiration decreased by the thiamine (p=0.026) and carnitine (p=0.008) treatments; this patient did not respond to these drugs. In Patient-3, respiration increased by the carnitine (p=0.012) treatment; the patient clinically responded to carnitine.

Conclusions: The results show cellular respiration is a suitable biomarker for the disease. The significance of using this tool to assess responses to therapies requires further studies.

Keywords: Cellular respiration; Fibroblasts; Lipomide dehydrogenase; DLD deficiency; Thiamine; Carnitine; Lipoate

Abbreviations:

ATP: Adenosine Triphosphate; Coa: Coenzyme A; CO_2: Carbon Dioxide; DLD: Dihydrolipoyl Dehydrogenase; dH_2O: Distilled Water; MEM: Minimum Essential Medium; NAD: Nicotinamide Adenine Dinucleotide; O_2: Oxygen; Pd: Palladium; PDHc: Pyruvate Dehydrogenase Complex; SD: Standard Deviation

Introduction

We recently reported on the use of phosphorescence oxygen analyzer to measure cellular respiration in fibroblasts, lymphocytes and foreskins from patients with inborn error of metabolism [1-3]. The same method is employed here to study fibroblast cellular respiration (mitochondrial O_2 consumption) in patients with dihydrolipoyl dehydrogenase (DLD) deficiency (MIM #238331). This autosomal recessive disorder results mostly from mutations in the DLD gene. Patients present with and early-onset lactic acidosis and developmental delay or late-onset neurological and hepatic dysfunctions [4]. These patients are routinely treated with combinations of DL-lipoic acid, thiamin and carnitine [5-7].

Lipomide dehydrogenase (EC 1.8.1.4; dihydrolipoyl dehydrogenase or E3 component) is a flavoprotein enzyme that oxidizes dihydroliopamide to lipoamide. E3 is an essential component of three related mitochondrial enzyme complexes involved in energy biotransformations, pyruvate dehydrogenase, α-ketoglutarate (or 2-oxoglutarate) dehydrogenase, and branched-chain α-keto acid dehydrogenase. Deficiency of E3, thus, is expected to impair cellular bioenergetics.

The pyruvate dehydrogenase complex couples glycolysis to the citric acid cycle; it catalyzes the pyruvate decarboxylation reaction that generates acetyl-CoA. The enzyme has three components, pyruvate dehydrogenase (EC 1.2.4.1, or E1 component; thiamine pyrophosphate is a co-enzyme of E1), dihydrolipoyl transacetylase (EC 2.3.1.12, or E2 component; lipoic acid is a co-enzyme of E2), and the E3 component (flavin adenine dinucleotide is a co-enzyme of E3).

The α-ketoglutarate dehydrogenase complex catalyzes a rate-limiting reaction in the citric acid cycle (α-ketoglutarate + NAD^+ + CoA → succinyl CoA + CO_2 + NADH). The enzyme has three components, α-ketoglutarate dehydrogenase (EC 1.2.4.2, or E1

component; thiamine pyrophosphate is a co-enzyme of E1), dihydrolipoyl succinyltransferase (EC 2.3.1.61, or E2 component; lipoic acid is a co-enzyme of E2), and the E3 component.

The branched-chain α-keto acid dehydrogenase complex catalyzes oxidative decarboxylations of L-leucine, L-isoleucine, L-valine, and their derivatives (including pyruvate). The enzyme has three components, α-ketoacid dehydrogenase (E1 component; thiamine pyrophosphate is a co-enzyme of E1), dihydrolipoyl transacylase (E2 component; lipoic acid is a co-enzyme of E2), and the E3 component [8].

Thus, the phosphate derivative of thiamine (thiamine diphosphate, also known as thiamine pyrophosphate) and the lipoyl moiety are essential catalytic co-factors in these critical energy-generating metabolic reactions. L-carnitine, on the other hand, is required for transport of fatty acids from the cytosol to the mitochondria as a source of energy. Therefore, known functions of the three compounds justify their administration to patients with lipomide dehydrogenase deficiency.

This study investigated the *in vitro* responses of fibroblast cellular respiration to lipoic acid, thiamine, and carnitine in patients with DLD deficiency. Our hypothesis was that these cofactors support cellular bioenergetics and improve respiration in patients with DLD deficiency.

Methods

Reagents

Pd (II) complex of *meso*-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin was purchased from Porphyrin Products (Logan, UT, USA). Pd phosphor solution (2.0 mg/ml = 2 mM) was prepared in dH_2O and stored at −20°C. Minimum Essential Medium (MEM Alpha Modification, #11900-016) was purchased from Gibco (Life Technology Corporation, Paisley, UK). Thiamine HCl Injection (100 mg/mL, 296.5 mM, *m.w.* 337.3) was purchased from APP Pharmaceuticals (Schaumburg, IL, USA). Levocarnitine Injection (200 mg/mL, 1.24 M, *m.w.* 161.2) and (±)-α-lipoic acid (*m.w.* 206.33) were purchased from Sigma-Tau Pharmaceuticals. The three compounds were stored at 4°C; appropriate dilutions were made in MEM and used immediately.

Fibroblasts

Tissue collection from all participants was approved by the institutional ethical review board for protection of human subjects. Informed consent was obtained for each patient. The fibroblast cultures were prepared from foreskin specimen of a normal infant and skin biopsies of three patients and processed as previously described [2].

Cells were treated at confluence with lipoic acid (50 or 100 μM), thiamine (233, 466, or 932 μM), or carnitine (100, 200, or 400 μM). Cells were harvested 24 hours after treatment and processed for measuring cellular respiration as previously described [1-3]. The drugs were used at saturating concentrations and there were no significant concentration-dependent effects. Therefore, the results for each compound were grouped.

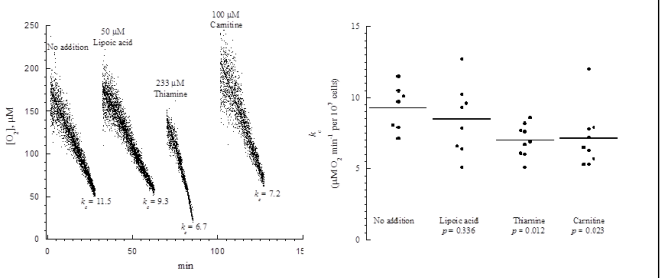
Data were analyzed using SPSS statistical package (version 20). The nonparametric test (2 independent variables; Mann-Whitney) was used to compare treated and untreated samples.

Results

Normal infant

The rate of cellular respiration (mean ± SD, in μM O_2 min^{-1} per 10^7 cells) in fibroblasts (passage #3) from a normal infant was 9.3 ± 1.6 (n=7). The corresponding rate in lipoic acid-treated fibroblasts was 8.0 ± 2.5 (n=8, p=0.336), thiamine-treated fibroblasts was 7.0 ± 1.1 (n=9, p=0.012), and carnitine-treated fibroblasts was 7.1 ± 2.1 (n=9, p=0.023), Figures 1A and 1B.

Feasible explanation of the lower rate of respiration with cofactors is more efficient oxidation of reduced metabolic fuels in the mitochondrial respiratory chain.



Figue 1: Cellular respiration in fibroblasts from a normal infant. Fibroblasts were treated at confluence with no addition or with the addition of lipoic acid, thiamine, or carnitine. The cells were harvested 24 hours after treatment and processed for measuring cellular respiration. The rate of respiration (k, μM $O2$ min^{-1}) was the negative of the slope of $[O_2]$ *vs. t.* (A) Representative O_2 measurements from seven to nine separate experiments for each condition are shown. The lines are best linear fit. The values of k_c (μM O_2 min^{-1} per 10^7 cells) are shown at the bottom of each run. (B) Summary of all measurements is shown. The concentrations of lipoic acid were 50 and 100 μM; thiamine 233, 466 and 932 μM; and carnitine 100, 200 and 400 μM. The results for each compound were grouped since there were no significant concentration-dependent effects. The horizontal lines are mean.

Patient 1

This infant presented at 24 hours of age with lactic acidosis (Table 1). Pyruvate dehydrogenase complex (PDHc) activity in his fibroblasts was severely reduced. Sequencing the DLD gene showed the homozygous c.1436A>T (p.Asp479Val) mutation. He was treated with thiamine (700 mg/day) and carnitine (100 mg/kg). Cellular respiration was measured in fibroblasts prepared from passage #3. Representative measurements of his fibroblast respiration in the presence of designated concentrations of lipoic acid, thiamine, and carnitine are shown in Figure 2A; a summary of all results is shown in Figure 2B.

The rate of cellular respiration (μM O_2 min^{-1} per 10^7 cells) without treatment was 5.1 ± 0.9 (n=6). The corresponding rates in the presence of lipoic acid, thiamine, and carnitine were 8.1 ± 1.8 (n=6, p=0.002), 8.5 ± 1.6 (n=9, p<0.001), and 7.6 ± 2.0 (n=9, p=0.018), respectively. Thus, his fibroblast respiration was significantly lower (p=0.001) than that of control fibroblasts, and all treatment conditions resulted in improved respiration. Clinically, this patient responded to thiamine.

Patient 3	Patient 2	Patient 1	
One year	Birth	Birth	Age at presentation
14 years	4 months	6 months	Age at diagnosis
16 years*	2 years	3 years	Age at study
Vomiting, seizure, hypoglycemia	Lactic acidosis	Respiratory distress, lactic acidosis	Presenting symptoms
Emirati	Palestinian	Palestinian	Nationality
Yes	Yes	Yes	Consanguinity
c.685 G>T (p.Gly229Cys)	c.1436A>T (p.Asp479Val)	c.1436A>T (p.Asp479Val)	Mutation
+	+++	++	Developmental impairment
-	+++	++	Hypotonia
Horizontal oculomotor apraxia	Nystagmus, not fixing or following	Normal	Eye
Right side severe sensorineural hearing loss	Conductive and sensorineural hearing loss	Normal	Hearing
?	Normal	Left ventricular hypertrophy	Cardiac involvement
No	No	Yes	Bilateral inguinal hernia
Yes	Yes	Yes	Lactic acidosis
++++	-	+	Elevated liver transaminase
Yes	No	No	Hyperammonemia
Normal$	↑Alanine	Normal	Plasma amino acid
Normal	?	Small basal ganglia infarcts	Brain MRI
Responded to intravenous glucose and carnitine	Not compliant with medications#	Carnitine, thiamine	Treatment

Table 1: Clinical features of the patients with DLD deficiency. *Patient died at 16 years of age as result of hyperammonemia and liver failure post viral infection. $Normal branched-chain amino acids; in one test, allo-isoleucine was 3 nmol/mL (reference values, 0-2). #Lactic acidosis initially improved with thiamine; patient developed fever after taking lipoic acid.

Patient 2

This infant also presented at birth with lactic acidosis (Table 1). PDHc activity in his fibroblasts was severely reduced. Sequencing DLD gene showed the same homozygous mutation c.1436A>T (p.Asp479Val). The rate of cellular respiration (μM O_2 min^{-1} per 10^7 cells) in early passages without treatment was 5.7 ± 1.4 (n=6) [2]. The rate of respiration in a late passage (passage #10) without treatment was 7.4 ± 1.4 μM O_2 min^{-1} per 10^7 cells (n=6). The corresponding rates in the presence of lipoic acid, thiamine, and carnitine were 6.6 ± 1.6 (n=6, p=0.180), 5.1 ± 1.4 (n=9, p<0.026), and 5.0 ± 1.7 (n=9, p=0.008), respectively (Figure 3). Thus, his fibroblast respiration in the early passages was significantly low (p=0.001) compared to control fibroblasts. In contrast, his fibroblast respiration in the later passage was similar to control fibroblasts (p=0.051) and significantly decreased with the treatments. Clinically, this patient had severe manifestations of the disease and did not respond to any of the drugs.

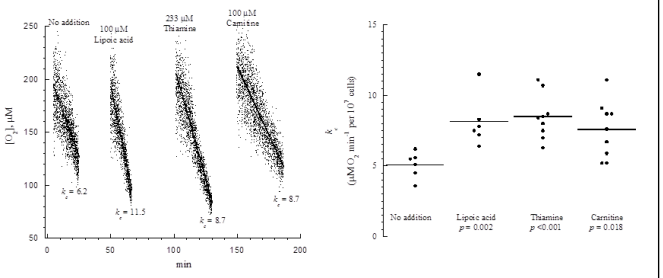


Figure 2: Cellular respiration in fibroblasts from Patient 1 (male infant with severely reduced fibroblast PDHc activity). Please see legend to Figure 1. (A) Representative O_2 measurements from six to nine separate experiments for each condition are shown. (B) Summary of all measurements is shown.

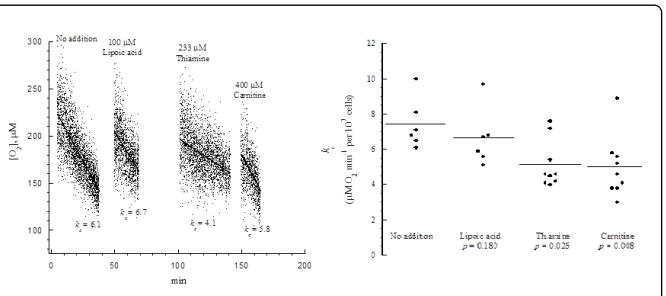


Figure 3: Cellular respiration in fibroblasts from Patient 2 (male infant with severely reduced fibroblast PDHc activity). Please see legend to Figure 1. (A) Representative O_2 measurements from six to nine separate experiments for each condition are shown. (B) Summary of all measurements is shown.

Patient 3

This 16-year-old male presented with infection-triggered vomiting, hypoglycemia and elevated hepatic transaminases. He had mild learning disability; otherwise, he was asymptomatic (Table 1). The PDHc activity in his fibroblasts was 10.9 mU/UCS (reference range, 9.7 to 36). Sequencing the *DLD* gene showed the homozygous mutation c. 685 G>T (p.Gly229Cys).

Cellular respiration in his fibroblasts (passage #8) is shown in Figure 4. The rate of respiration (μM O_2 min^{-1} per 10^7 cells) without treatment was 10.3 ± 3.3 μM O_2 min^{-1} per 10^7 cells (n=6). The corresponding rates in the presence of lipoic acid, thiamine, and carnitine were 12.0 ± 2.3 (n=6, p=0.394), 13.6 ± 4.1 (n=9, p<0.181), and 15.5 ± 2.8 (n=9, p=0.012), respectively. Thus, although his fibroblast respiration was normal (p=0.836), it was significantly

improved with the carnitine treatment. Clinically, this patient responded to carnitine.

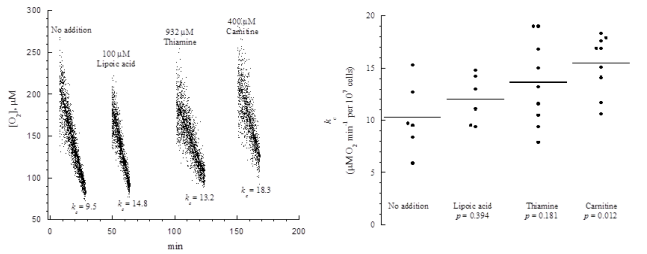


Figure 4: Cellular respiration in fibroblasts from Patient 3 (adolescent male with normal fibroblast PDHc activity). Please see legend to Figure 1. (A) Representative O_2 measurements from six to nine separate experiments for each condition are shown. (B) Summary of all measurements is shown.

Discussion

The term "cellular bioenergetics" is defined as the biochemical processes involved in energy transformation, and the term "cellular respiration" is defined as the processes of delivering nutrients (catabolic metabolic fuels) and O_2 to the mitochondria, oxidation of reduced metabolic fuels, passage of electrons to O_2, and synthesis of ATP. Therefore, impairments in any of these processes will interfere with the rate of cellular respiration. Thus, DLD deficiency is expected to impair cellular bioenergetics and respiration [6-7].

Reference values for fibroblast respiration were recently reported [2]. The rate was 9.8 ± 2.4 µM O_2 min^{-1} per 10^7 cells (median=10.5, range=6.6 – 14.3, n=15). The two infants (Patients 1 and 2) with the homozygous mutation c.1436A>T had early-onset disease with severely reduced PDHc activities, but Patient 2 had a more severe phenotype than Patient 1. Their fibroblast respiration in early passages (#3) were also reduced (p=0.001). In Patient 1, the fibroblast respiration was significantly increased with all tested compounds (Figure 2). In Patient 2, the fibroblast respiration in the late passage (#10) was similar to control (p=0.051) and not increased with treatment (Figure 3). It is worth noting that the *in vitro* testing should be performed in the same cell culture passage, ideally in early passages (#3).

Patient 3 had a milder disease with normal PDHc activity, consistent with his homozygous mutation (c.685G>T) [4]. His fibroblast respiration was normal in the fourth and sixth cell culture passages, but the lymphocyte respiration was low (data not shown). Thus, for disease screening, testing different tissues is recommended.

Lymphocyte respiration was measured in four patients with pyruvate dehydrogenase complex deficiency (Patients 1 and 2, and two other patients); the rate was 1.0 ± 0.62 µM O_2 min^{-1} per 10^7 cells. The reference rate in 20 healthy children was 2.0 ± 0.9 µM O_2 min^{-1} per 10^7 cells (median=2.0, and range = 0.9 – 3.7) [1,2]. Thus, lymphocyte respiration was reduced (p=0.067) in these patients compared to the controls.

It is worth noting that interacting metabolic reactions are necessary to maintain stability of the cellular bioenergetics. These processes require coordinating anabolic pathways in the nucleus and catabolic pathways in the mitochondria to create steady state energy potential in the cytosol [9]. As previously suggested, inborn errors of metabolism impose imbalance in the catabolic and anabolic processes, which results in human diseases [9]. In addition, mitochondrial dysfunction may result from caspase activation, which impairs cellular bioenergetics. However, this dependency on aerobic metabolic pathways may not apply to cells (e.g., cancer cells) that can maintain their survival on anaerobic metabolism ("aerobic glycolysis" or Warburg effect) [10]. Thus, the biophysical changes in cellular bioenergetics ("capacitor operations") in DLD deficiency and its clinical implications are expected to be complex and require investigations in future studies [9].

Conclusion

The results show cellular respiration is a suitable biomarker for DLD deficiency. The significance of using this tool to assess responses to therapies requires further studies. This approach may allow testing novel agents for disease treatment.

Competing Interest

None

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