HHS Public Access

Author manuscript

Am J Med Genet A. Author manuscript; available in PMC 2021 October 25.

Published in final edited form as:

Am J Med Genet A. 2020 October ; 182(10): 2426–2431. doi:10.1002/ajmg.a.61786.

Mitochondrial energetic impairment in a patient with late-onset glutaric acidemia Type 2

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Abstract

Glutaric acidemia type 2 (GA2), also called multiple acyl-CoA dehydrogenase deficiency, is an autosomal recessive disorder of fatty acid, amino acid, and choline metabolism resulting in excretion of multiple organic acids and glycine conjugates as well as elevation of various plasma acylcarnitine species (C4–C18). It is caused by mutations in the *ETFA*, *ETFB*, or *ETFDH* genes which are involved in the transfer of electrons from 11 flavin-containing dehydrogenases to Coenzyme Q_{10} (CoQ_{10}) of the mitochondrial electron transport chain (ETC). We report a patient who was originally reported as the first case with primary myopathic CoQ_{10} deficiency when he presented at 11.5 years with exercise intolerance and myopathy that improved after treatment

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AUTHOR CONTRIBUTIONS

Drs Kimberly A. Chapman, Jerry Vockley, and Carlos R. Ferreira contributed to the conception and design of this study. Dr Fernando Scaglia, Dr Kimberly A. Chapman, Ms Monisha Kisling, Ms Shrabani Basu, Dr Yudong Wang, Dr Jerry Vockley, and Dr Carlos R. Ferreira contributed to the acquisition of the data. Dr Changrui Xiao, Dr Esteban Astiazaran-Symonds, Ms Shrabani Basu, Dr Yudong Wang, Dr Jerry Vockley, and Dr Carlos R. Ferreira contributed to analysis and interpretation of the data. Dr Changrui Xiao, Dr Esteban Astiazaran-Symonds, Ms Shrabani Basu, Dr Jerry Vockley, and Dr Carlos R. Ferreira contributed to drafting the manuscript. All authors contributed to revising the manuscript for critical intellectual content.

CONFLICT OF INTEREST

The authors of this manuscript have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

with ubiquinone and carnitine. At age 23, his symptoms relapsed despite increasing doses of ubiquinone and he was shown to have biallelic mutations in the *ETFDH* gene. Treatment with riboflavin was started and ubiquinone was changed to ubiquinol. After 4 months, the patient recovered his muscle strength with normalization of laboratory exams and exercise tolerance. Functional studies on fibroblasts revealed decreased levels of ETFDH as well as of very long-chain acyl-CoA dehydrogenase and trifunctional protein α. In addition, the mitochondrial mass was decreased, with increased formation of reactive oxygen species and oxygen consumption rate, but with a decreased spared respiratory capacity, and decreased adenosine triphosphate level. These findings of widespread dysfunction of fatty acid oxidation and ETC enzymes support the impairment of a larger mitochondrial ETC supercomplex in our patient.

Keywords

electron transport chain; ETFDH; glutaric acidemia type 2; mitochondria

1 | INTRODUCTION

Glutaric acidemia type 2 (GA2), also called multiple acyl-CoA dehydrogenase deficiency (MADD, MIM #231680), is an autosomal recessive disorder caused by pathogenic variants in *ETFA*, *ETFB*, or *ETFDH* genes that encode the subunits of electron transfer flavoprotein (ETF) or ETF dehydrogenase (ETFDH), also known as ETF-ubiquinone oxidoreductase. Mutations in the genes for riboflavin transport and metabolism can also overlap in clinical and biochemical features. Pathogenic variants impair the transfer of electrons from 11 flavin-containing dehydrogenases to coenzyme Q_{10} (CoQ_{10}) of the mitochondrial electron transport chain (ETC; Bell et al., 1990). ETFDH is a mitochondrial protein localized in the inner mitochondrial membrane that mediates electron transport from flavoprotein dehydrogenases to the ubiquinone pool (Liang et al., 2009; Zhang, Frerman, & Kim, 2006). Pathogenic variants in *ETFDH* result in impaired adenosine triphosphate (ATP) biosynthesis, excessive lipid accumulation in different organs and insufficient gluconeogenesis (Grünert, 2014). Recent data suggest ETFDH is part of a multiprotein energy complex, along with some of its functional partners including very long-chain acyl-CoA dehydrogenase (VLCAD) as well as the alpha and beta subunits of Trifunctional Protein (TFPα and TFPβ), where it has been shown to interact with the CoQ_{10} containing core 2 subunit of Complex III of the ETC (Wang et al., 2019).

Patients with GA2 have abnormal fatty acid, amino acid, and choline metabolism. Biochemical diagnosis is based on the urinary organic acid profile and the acylcarnitine pattern in dried blood/plasma. The characteristic urine organic acid pattern is an elevation of glutaric, ethylmalonic, adipic, 3-hydroxyisovaleric, 2-hydroxyglutaric, 5-hydroxyhexanoic, suberic, sebacic, and dodecanedioic acids without relevant ketonuria, especially if combined with glycine conjugates of C4 and C5 acids. Blood acylcarnitine profile usually reveals increased concentrations of several short-, medium-, and long-chain acylcarnitines, such as C4, C5, C5-DC, C6, C8, C10, C12, C14:1, C16, and C18:1 (Amendt & Rhead, 1986; Boyer, Barclay, & Burrage, 2015).

Clinical features of this disorder fall into a continuum of symptoms traditionally classified into three types: neonatal with congenital anomalies (Type 1), neonatal without congenital anomalies (Type 2) and late-onset (Type 3). Neonatal onset forms usually present with hypotonia, hepatomegaly, hyperammonemia, nonketotic hypoglycemia, metabolic acidosis, and poor prognosis. Milder Type 3 patients manifest proximal myopathy often with hepatomegaly, encephalopathy, and episodic lethargy, vomiting and hypoglycemia. In the mildest forms of GA2, muscle weakness developing in adulthood may be the first sign of the disorder (Boyer et al., 2015; Frerman & Goodman, 1985).

In this study, we report the clinical, molecular, and functional findings of a 23-year-old man affected by late-onset GA2 that presented fluctuating weakness since childhood, previously thought to have a primary defect in CoQ_{10} synthesis.

2 | METHODS

2.1 | Editorial policies and ethical considerations

The patient was consented to research protocol Pro00004911, “Bio-repository for cells, tissues and DNA”. This study was approved by the Institutional Review Board at Children’s National Health System.

2.2 | Cell culture

Fibroblasts were cultured from skin biopsy in DMEM media (Corning Life Sciences, Tewksbury, MA) supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 IU penicillin, and 100 μg/ml streptomycin (Corning Life Sciences) at 37°C in 5% CO_2. Control fibroblasts were from age- and sex-matched controls with similar passage number to patient cells. All samples were assayed with replicates of $n = 6$. Means and *SD* were calculated using the instrument software. Differences for each parameter were compared between control and patient cell values and their statistical significance was determined using an unpaired t test from a single biological replicate in Prism GraphPad software v 7.0.

2.3 | Western blot

Patient cell culture (FB862) and control cell culture (FB826), were grown to 90–95% confluence, and cells were harvested by trypsinization. Cell-free extract was made by using RIPA buffer and protease inhibitor cocktail (Sigma, Santa Fe, NM). Protein estimation of cell-free extract was done using DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Total of 10–40 μg of protein was loaded on the gel for Western blotting. Membranes were probed with antibodies to ETFDH (SC515202, Santa Cruz Biotechnologies Inc., Dallas, TX); VLCAD (SC271225); core protein 2 of Complex III (ab203832, Abcam, Cambridge, UK); TFPα and TFPβ (custom-made, Cocalico Biologicals Inc., Reamstown, PA); and GAPDH (ab8245). Protein levels were quantified by densitometric analysis of scanned blots with GAPDH as a control using NIH ImageJ software.

2.4 | Mitochondrial superoxide levels and mitochondrial mass

Three hundred thousand patient and control cells were incubated for 20 min at 37°C with 5 μM MitoSOX Red (Invitrogen, Carlsbad CA) for superoxide production assessment, or

with 150 nM MitoTracker Green (Invitrogen) for mitochondrial mass evaluation. After incubation, 10,000 cells were analyzed in a FACSAriaII Flow cytometer (BD Bioscience, San Jose, CA). Data shown are calculated in GraphPad Prism 7 using an unpaired t test for samples from a single biological replicate and minimum of at least three technical replicates.

2.5 | ATP level

Steady-state level of ATP were measured with a bioluminescence assay kit (ATPlite, Perkin Elmer Inc., Waltham, MA), according to the manufacturer's instructions. Luminescence was quantitated in a SpectraMax i3x Platform multimode microplate reader system (Molecular Devices, San Jose, CA). Data are reported as μmol of ATP/mg of cellular protein.

2.6 | Measurement of mitochondrial respiration

Cellular oxygen consumption rate (OCR) was measured using a Seahorse XFe96 Extracellular Flux Analyzer (Agilent, Santa Clara, CA). Cells were seeded in 96-well Seahorse tissue culture microplates in growth media at a density of 80,000 cells per well. Cells were counted using a Cell Countess Kit (Invitrogen), and an equal number of cells were seeded in cell culture-treated plates. All cultured fibroblasts were measured with eight wells per cell line as described (Frerman & Goodman, 1985). Data are reported as pmol of O_2 reduced/min. Statistical analysis was performed with GraphPad Prism 7 using an unpaired t test for samples from a single biological replicate and a minimum of seven technical replicates.

3 | RESULTS

Our patient was originally reported as the first case with primary myopathic CoQ_{10} deficiency (Lalani et al., 2005). He presented at 11.5 years of age with slowly progressive exercise intolerance accompanied by predominantly lower proximal muscle weakness and pain. Development was normal and family history was non-consanguineous with no other neuromuscular or metabolic diseases. Laboratory studies showed elevation in serum creatine kinase and lactate. Urine organic acid analysis detected abnormal metabolites including ethylmalonic acid, methylsuccinic acid, hexanoylglycine, and lactic acid. The plasma acylcarnitine profile exhibited elevations of butyrylcarnitine, pentanoylcarnitine, hexanoylcarnitine, octanoylcarnitine, and decanoylcarnitine, with no evidence of plasma carnitine depletion. Skeletal muscle histochemical studies were notable for ragged-red fibers, reduction in Complex I, I + III, and II + III activity, and a CoQ_{10} concentration that was 46% of the reference range. In addition, in vitro studies of-skeletal muscle whole homogenate showed improved succinate cytochrome-c reductive activity compared to control samples in response to addition of CoQ_{10}. Empirical treatment with ubiquinone and carnitine were initiated, which led to normalization of creatine kinase and lactate levels, as well as clinical improvement in endurance and strength, including the ability to perform routine activities such as walking between classes without difficulty.

At 23 years of age, his muscle weakness, myalgia, and extreme fatigue relapsed, limiting head elevation and reducing his exercise tolerance to a few steps. On evaluation, creatine kinase was elevated to 744 U/L (normal 22–198 U/L), aspartate transaminase was elevated

to 271 U/L (normal 10–40 U/L), and alanine transaminase was elevated to 660 U/L (normal 7–56 U/L), with suggestion of fatty liver on abdominal ultrasound. His workup for liver disease included ceruloplasmin, hepatitis B surface antigen, hepatitis C antibody, ferritin, and iron studies which were normal. Echocardiogram showed normal left ventricular size and systolic function. His electrocardiogram showed sinus tachycardia. Blood acylcarnitine profile showed elevation of multiple acylcarnitine species, especially C14–C18, which was originally interpreted as consistent with a long-chain fatty acid oxidation disorder. However, urine organic acids showed elevations of ethylmalonic acid, isovalerylglycine, glutaric acid, and hexanoylglycine, consistent with a diagnosis of GA2 (Supplemental Table S1). Sequencing of the *ETFDH* gene revealed one known pathogenic mutation (c.665A > C; p.Gln222Pro) and one variant of unknown significance (c.964G > T; p.Gly322Cys) confirmed to be *in trans*. The latter was not found in gnomAD, is highly conserved across species, and predicted to be pathogenic by common in silico algorithms including SIFT and Polyphen-2, with a Phred-scaled CADD score of 33 (Rentzsch, Witten, Cooper, Shendure, & Kircher, 2019). The p.Gln222Pro variant is predicted to be in the FAD-binding domain while the p.Gly322Cys variant is predicted to be in the ubiquinone-binding domain of ETFDH (Missaglia, Tavian, Moro, & Angelini, 2018). Parental testing revealed that the patient's mother is a carrier for the Gln222Pro variant while his father carries the Gly322Cys variant.

Riboflavin supplementation was started at 100 mg daily and then twice daily, and ubiquinone was changed to ubiquinol. After 4 months, the patient had experienced recovery with normalization of his laboratory exams, exercise tolerance, and self-reported normalization of performance status and endurance, including the ability to walk more than a mile without fatigue or shortness of breath.

Western blot demonstrated decreased ETFDH, TFPα, and VLCAD protein bands when normalized to GAPDH relative to controls (Figure 1a–c). ETFDH was decreased by 73%, TFPα was decreased by 48%, and VLCAD was decreased by 31%. TFPβ was unchanged or minimally reduced. Mitochondrial superoxide was increased in patient compared to control cells ($p < .0001$) while mitochondrial mass was decreased ($p < .01$) (Figure 2a,b). Basal OCR in patient cells was significantly increased ($p < .001$), while spare respiratory capacity (defined as the maximal respiration—basal respiration) was decreased (Figure 2c,d). Steady-state ATP level in patient cells was decreased compared to control cells ($p < .0001$) (Figure 2e).

4 | DISCUSSION

We provide follow-up information on an individual previously reported with isolated myopathy from primary CoQ_{10} deficiency, now demonstrated to have GA2 due to biallelic *ETFDH* variants. Functional testing demonstrated decreased levels of ETFDH, VLCAD, and TFPα protein as well as decreased mitochondrial mass, increased oxidative stress, and decreased cellular ATP levels. These results provide functional data that allow us to interpret the c.964G > T variant as likely pathogenic by the American College of Medical Genetics and Genomics criteria. (Richards et al., 2015) We also report preliminary evidence of global mitochondrial bioenergetics dysfunction and protein expression changes as contributing factors to GA2 pathophysiology in addition to canonical electron transfer duties of ETFDH.

While a decreased ETFDH level may be explained by reduced *ETFDH* expression, a more general decrease in the levels of other members of the multienergy protein complex (VLCAD and TFPα) suggests reduced stability of the complex (Wang et al., 2019). It is noteworthy that while most TFPα or TFPβ mutations affect the stability of both subunits, this patient had essentially an isolated reduction in TFPα protein. We hypothesize that it is related to a balance between the relative position of TFP in the multiprotein energy complex and its membrane interaction; however, the dynamics of protein association in this complex are still poorly understood. Enzyme activity of these proteins was not measured. Reduction of these proteins demonstrates the need to consider potential secondary effects of primary defects in members of this complex. Decreased total mitochondrial mass could be related to decreased global expression of nuclear mitochondrial genes, impaired mitochondrial biogenesis, and/or reduced mitochondrial stability as a contributing factor to the biochemical derangements seen in patient cells.

Additional studies revealed increased reactive oxygen species (ROS), decreased spare respiratory capacity, increased basal oxygen consumption, and decreased steady-state cellular ATP level consistent with mitochondrial dysfunction and energy homeostasis impairment. Previous studies in cells of patients with ACAD9 related Complex I deficiency (Leipnitz et al., 2018) and VLCAD deficiency (Seminotti et al., 2019) found similar results. ATP synthesis is likely reduced due to respiratory chain supercomplex instability as well as reduced flux of reducing equivalents from ETFDH to ETC Complex III. The former phenomenon is likely due to physical disruption of the macromolecular FAO/ETC energy complex. This can lead to both impaired oxidative phosphorylation as well as increased ROS that feedback and further impair the ETC supercomplexes. The increase in OCR and decrease in ATP levels suggest a degree of uncoupling. Prior studies demonstrated increased Uncoupling Protein 3 expression in muscle from patients with MADD (Russell et al., 2003), and uncoupling effects of many accumulated intermediates in fatty acid oxidation defects (Wajner & Amaral, 2015).

We hypothesize that impaired interaction of a mutant ETFDH with the CoQ10 containing core 2 protein of ETC Complex III may explain the original classification of this patient as having a primary defect in CoQ_{10}. Since CoQ_{10} is the direct acceptor of electrons from flavins mediated by ETFDH and is important for Complex III stability (Acín-Pérez, Fernández-Silva, Peleato, Pérez-Martos, & Enriquez, 2008), both impaired electron transfer and reduced structural integrity in the inner mitochondrial enzyme complex are potential mechanisms of GA2 pathophysiology and the secondary CoQ_{10} deficiency in our patient. This finding may be mediated by either downregulation of CoQ_{10} biosynthesis and/or its excessive degradation. This case also supports late-onset GA2 and the myopathic form of CoQ_{10} deficiency may be allelic diseases, especially as muscle histology on previous reported individuals with a myopathic CoQ_{10} deficiency phenotype found to have pathogenic variants in *ETFDH* showed signs of mitochondrial myopathy (Gempel et al., 2007).

Riboflavin is a precursor to FAD, in turn a cofactor for ETF, and there is evidence FAD is able to correct chaperone-mediated folding defects as well as stabilize the conformation of variant ETF protein caused by *ETFDH* missense variants (Olsen et al., 2007). Therefore,

riboflavin supplementation was initiated in the patient. Since ETFDH enzyme donates electrons to reduce ubiquinone into ubiquinol as the substrate for Complex III, the novel variant in our patient is in the ubiquinone-binding domain, we continued CoQ_{10} therapy though the formulation was changed from oral ubiquinone to ubiquinol. Oral ubiquinone is reduced to ubiquinol in enterocytes upon absorption, and ubiquinol appears to have greater bioavailability (Langsjoen & Langsjoen, 2014). This patient's clinical response suggests the variant ETFDH led to an inability to generate enough ubiquinol and Complex III function was preserved. Another mechanism for improvement is likely stabilization of the core 2 protein, and ultimately the multiprotein energy complex, perhaps due to better bioavailability, though this was not formally tested. Since both riboflavin and ubiquinol were started simultaneously, we do not know the extent our patient would have responded to riboflavin or ubiquinol monotherapy. A study of riboflavin-responsive MADD cases with *ETFDH* mutations found increased CoQ_{10} levels by HPLC in muscle (Wen et al., 2013), which is contrary to the findings in our patient. In a previously reported cohort of patients with myopathic CoQ_{10} deficiency due to *ETFDH* mutations, patient response to riboflavin monotherapy varied (Gempel et al., 2007). Additionally, treatment with CoQ_{10}, but not with riboflavin, has been shown to normalize the concentration of CoQ_{10} in patient cells (Cornelius et al., 2013).

The pathogenesis of GA2 is traditionally thought to be due to disruption of the electron transfer from and reoxidation of the primary FAD-containing mitochondrial dehydrogenases leading to their inactivity. We find evidence of including mitochondrial dysfunction, increased oxidative stress, and decreased stability of the mitochondrial multiprotein energy Complex In our patient. Further study is necessary to better understand how pathogenic variants in *ETFDH* affects each of these functions and whether these findings are generalizable in GA2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding information

National Institute of Diabetes and Digestive and Kidney Diseases, Grant/Award Number: K08DK105233

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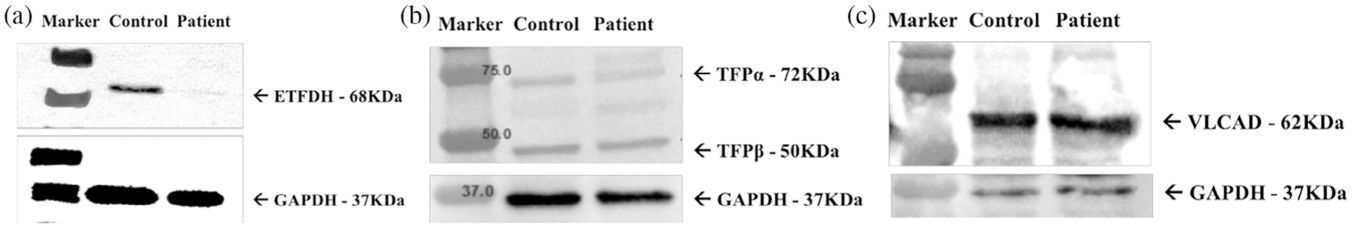

FIGURE 1.

Concentration of ETFDH, very long-chain acyl-CoA dehydrogenase (VLCAD), trifunctional protein (TFPα), and TFPβ protein relative to GAPDH control in cells from our patient versus healthy control. (a) ETFDH protein (upper panel) concentration was decreased by 73% as compared to control. Cell free extract (CFE) 10 μg loaded in lanes marked as Control and ETFDH-deficient (ETFDHD). Lower panel shows GAPDH as loading control. (b) Compared to control, protein levels of TFPα and β both diminished (48 and 10%, respectively) in ETFDHD patient CFE, 40 μg loaded. Lower panel shows GAPDH as loading control. (c) Compared to control, VLCAD diminished 31% in ETFDHD patient CFE, 40 μg loaded. Lower panel shows GAPDH as loading control

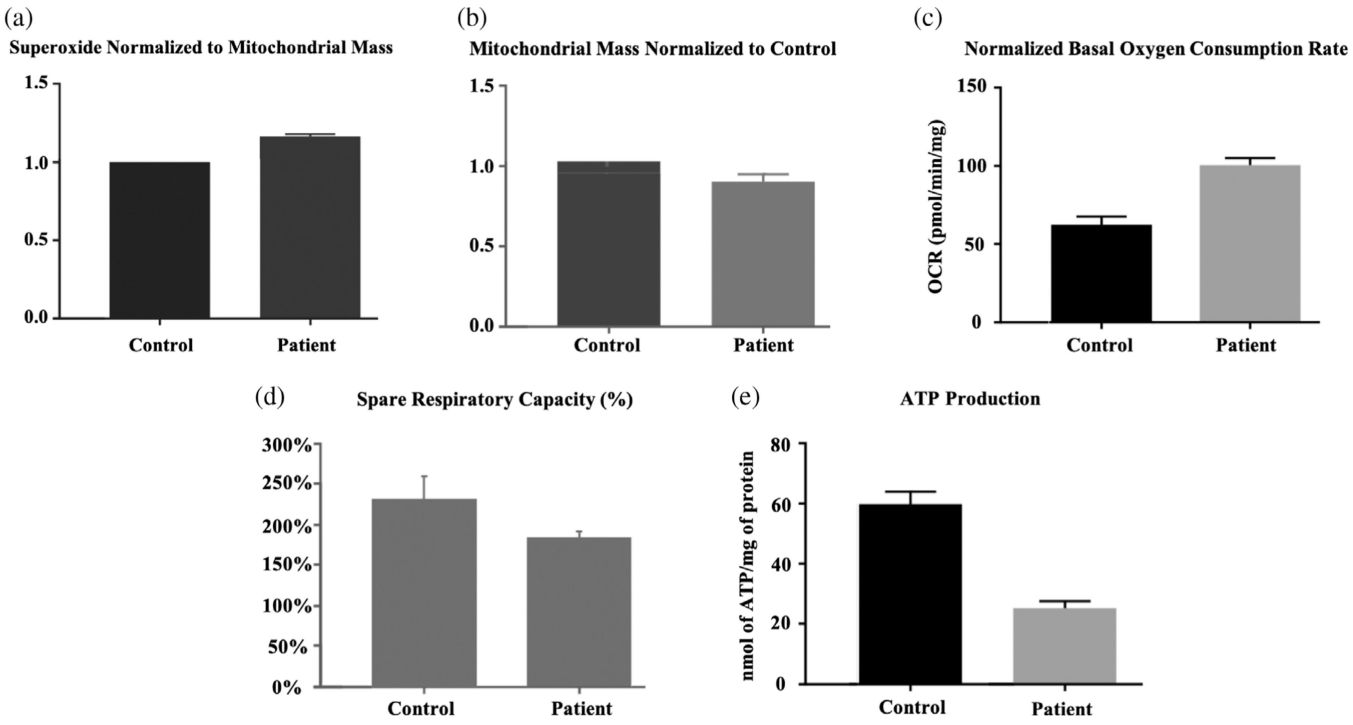

FIGURE 2.

Measurements of various markers of mitochondrial function in cells from our patient versus healthy control. (a) Superoxide levels detected by MitoSOX Red assay in patient and control cells. Data shown here are means ± *SD* and are normalized to mean value of control cells. ****$p < .0001$. (b) Mitochondrial mass detected by MitoTracker Green assay in ETFD-deficient and healthy control cells. Data shown here are means ± *SD* and are normalized to mean value of control cells. **$p < .01$. (c) Mitochondrial basal respiration of healthy control compared to ETFDHD fibroblast cell line. Data shown here are means ± SEM and are normalized to protein amount mean value from a single biological replicate and seven technical replicates. ***p value <.001. (d) Mitochondrial spare respiratory capacity of healthy control compared to ETFDHD fibroblast cell line. Data shown here are means ± *SD* and are normalized to protein amount mean value from a single biological replicate and seven technical replicates. (e) Mitochondrial adenosine triphosphate (ATP) of ETFDHD (FB862) compared to healthy (FB826) fibroblast cells. Data shown here are means ± *SD* and are normalized to protein amount mean value from a single biological replicate and four technical replicates. ****p value <.0001