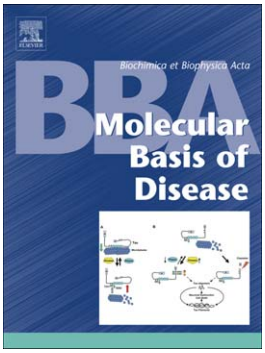

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The addition of ketone bodies alleviates mitochondrial dysfunction by restoring complex I assembly in a MELAS cellular model

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Abbreviations

Complex I, CI; Oxidative Phosphorylation, OXPHOS; mtDNA, mitochondrial DNA; NADH Dehydrogenase, ND; Reduced Nicotinamide Adenine Dinucleotide Hydrate, NADH; Nicotinamide Adenine Dinucleotide, NAD^+; Reactive Oxygen Species, ROS; Kilo Dalton, kDa; Blue Native PAGE, BN-PAGE; HG, High Glucose; KB, ketone bodies; TCA, tricarboxylic acid cycle; HG, High Glucose

Abstract

Ketogenic Diet was used to treat refractory epilepsy for almost a century may represent a treatment option for mitochondrial disorders for which effective treatments are still lacking. Mitochondrial complex I deficiencies are involved in a broad spectrum of inherited diseases including Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes syndrome leading to recurrent cerebral insults resembling strokes and associated with a severe complex I deficiency caused by mitochondrial DNA (mtDNA) mutations.

The analysis of MELAS neuronal cybrid cells carrying the almost homoplasmic m.3243A>G mutation revealed a metabolic switch towards glycolysis with the production of lactic acid, severe defects in respiratory chain activity and complex I disassembly with an accumulation of assembly intermediates. Metabolites, NADH/NAD^+ ratio, mitochondrial enzyme activities, oxygen consumption and BN-PAGE analysis were evaluated in mutant compared to control cells. A severe complex I enzymatic deficiency was identified associated with a major complex I disassembly with an accumulation of assembly intermediates of 400 kDa. We showed that Ketone Bodies (KB) exposure during 4 weeks associated with glucose deprivation significantly restored complex I stability and activity, increased ATP synthesis and reduced the NADH/NAD+ ratio, a key component of mitochondrial metabolism. In addition, without changing the mutant load, mtDNA copy number was significantly increased with KB, indicating that the absolute amount of wild type mtDNA copy number was higher in treated mutant cells. Therefore KB may constitute an alternative and promising therapy for MELAS syndrome, and could be beneficial for other mitochondrial diseases caused by complex I deficiency.

Keywords: Mitochondria; Mitochondrial diseases; Mitochondrial DNA; MELAS syndrome; Complex I assembly; Ketone bodies.

1. Introduction

Mitochondria are responsible for the production of ATP through oxidative phosphorylation (OXPHOS) required for cellular functions. Mitochondrial diseases may result from mutations in either the nuclear genome or the mitochondrial DNA (mtDNA). Mitochondrial Encephalomyopathy, Lactic Acidosis and Strokes like episodes (MELAS) syndrome is characterized by a triad of symptoms, which are encephalopathy, stroke like episodes before the age of 40 and lactic acidosis [1]. Other clinical features include at various degrees other neurological symptoms, exercise intolerance, cardiomyopathy, deafness and diabetes. About 80% of MELAS syndromes are caused by the m.3243A>G mutation in the mtDNA resulting in a substitution in the $tRNA^{leu(UUR)}$ gene [2]. Furthermore, the severity of the disease is determined by the heteroplasmic rate which is the ratio of mutant to normal mtDNA [3].

MELAS syndrome is also associated with lactic acidosis and commonly associated with multiple partial respiratory chain defects, especially involving complex I and/or complex IV deficiencies [1]. Complex I defect appears as a prevalent feature in MELAS syndrome [4]. Moreover, a close relationship appears to exist between MELAS and complex I deficiency as reported into the literature [4-6]. In addition, complex I deficiency is present in up to 30% of mitochondrial diseases [7] and in neurodegenerative diseases such as Parkinson's disease, for which effective curative therapies are still lacking [8].

Therapies for mitochondrial diseases are still limited because of the rarity and the clinical heterogeneity of these diseases. The so called "mitochondrial cocktails" including carnitine, antioxidants or coenzyme Q10 are commonly used in mitochondrial disorders, including MELAS, but consensus for its use and efficacy are missing [8]. L-arginine or even citrulline have been proposed as a treatment for stroke-like episodes of MELAS owing to the fact that vascular endothelial cell vasodilator NO is reduced in this disease [9, 10]. However, the discrepancy between the generalized energetic defect observed in MELAS and the specific neurovascular

clinical expression remains largely unexplained [11]. Several other pathophysiological mechanisms including impaired mitochondrial energy production or microvasculature angiopathy are involved in MELAS and may require additional therapeutic strategies.

The ketogenic diet composed of a high fat and low carbohydrate regimen, has been used since the last century to treat refractory epilepsy and is usually based on lipid:nonlipid ratios of either 3:1 or 4:1 (for review see [12]). Very recently, a Cochrane study on ketogenic diet used for intractable epilepsies judged only seven randomized controlled clinical trials on pediatrics patients to be methodologically valid. All randomized trials showed promising results supporting the use of ketogenic diet in epilepsy [13]. The regimen has also shown beneficial effects in patients with mitochondrial respiratory chain defects [14], or Leigh syndrome [15]. The idea beyond this treatment is to substitute another energetic substrate than glucose to the mutant cells. Basically, the fatty acids are used in the liver to produce ketones bodies, mainly acetoacetate and β-D-hydroxybutyrate, which are able to pass the blood brain barrier and switch from glycolysis to the use of ketone bodies (KB) fuelling directly mitochondria. It was further hypothesised that KB used as substrates for complex II, increase the oxidative metabolism bypassing complex I [16, 17].

To investigate the metabolic mechanisms of KB in MELAS syndrome, a transmitochondrial mutant cybrid model shown to be associated with a severe complex I deficiency was treated over a period of 4 weeks demonstrating a significant improvement of complex I assembly and activity.

2. Material and Methods

2.1. Cell culture

The SH-SY5Y parental cells and mutant cybrid cells carrying the m.3243A>G with 98.6% mutant load were cultured in standard DMEM high glucose media (4.5 g/L) or in low glucose (0.5g/L) (PAN biotech), supplemented with 10% foetal bovine serum, 1% glutamine and 50µg/ml uridine (Sigma) at 37°C in presence of 5% CO2 as described elsewhere [18]. The medium was changed every 3 days during 4 weeks. The doses of KB doses were chosen in agreement with concentrations previously used in *in vitro* studies [19]. The duration of the KB exposure and time course and cell collection were selected based on our previous study [18]. For the KB treatment, 5 mM of acetoacetate and β-D-hydroxybutyrate (Sigma Aldrich, Lyon, France) were added to the low glucose media, and cells collected after 14 or 28 days for further analysis.

2.2. Quantification of mtDNA heteroplasmy and mtDNA copy number

The quantification of the mutant load was performed as described [18]. To determine the mtDNA copy number in cells, quantitative PCR (Q-PCR) was performed by SYBR Green incorporation using the Chromo4 System (Biorad) in a 20 ul reaction volume containing a final concentration of 0.5 mM of each gene-specific primer. The pairs of selected primers were, respectively, ND4 (Forward: actctcactgcccaagaact and Reverse: gtgtgaggcgtattatacca) and COX1 (Forward: tacgttgtagcccacttccact and Reverse: agtaacgtcggggcattccg) to quantify the mtDNA copy number, and B2 microglobulin (Forward: cagcctattctgccagcc and Reverse: caatgttctccacatagtgaggg) to quantify the nuclear DNA in mutant and control SHSY-5Y cells. The reactions were performed as follows: initial denaturing at 95C for 10 min, and 35 cycles at 95C for 30 s, 60C for 1 min. A melting curve was analyzed in order to check the specificity of the PCR products.

2.3. Metabolites and NADH/NAD$^+$ measurements

Glucose, pyruvate and lactate concentrations were measured by spectrophotometry with a Xenius apparatus (SAFAS, Monaco) according to the manufacturer (Abcam, Paris France). Acetoacetate concentration was determined by measuring the NADH oxidation at pH 7 by the β-hydroxybutyrate dehydrogenase enzyme (Roche Applied Science, Penzberg Germany). β-D-hydroxybutyrate concentration was determined by measuring the NADH reduction by the β-hydroxybutyrate dehydrogenase enzyme. The cytosolic NAD(H)-redox state was determined using the metabolite indicator method, based on the lactate dehydrogenase reaction, by measuring pyruvate to lactate ratio in cell culture media.

2.4. Mitochondrial enzymatic activities and oxygen consumption measurements

Activities of OXPHOS complexes and citrate synthase were measured at 37°C on an UVmc2 spectrophotometer (SAFAS, Monaco) as described [18]. The O_2 consumption was measured using an Oroboros oxygraph (Oroboros, Austria) on both intact cells according to[20] and permeabilized cells, using digitonin [21].

2.5. Blue-Native-PAGE of respiratory chain complexes

Mitochondria were extracted using digitonin and complex assembly was revealed using antibodies against the NDUFS2 complex I subunit and the 70 kDa complex II subunit as a loading control (Abcam, Paris France), and visualized using an enhanced chemiluminescence kit (Pierce, Waltham USA) on a Li-cor ODYSSEY-Fc apparatus (Li-Cor Biosciences Biotechnology, Lincoln USA) [18].

2.6. Statistical analysis

Differences between groups were evaluated by the Mann-Witney statistical test for independent samples and by the Wilcoxon signed rank test for related samples. The asterisk (*, ** and ***) indicates significant differences (respectively $p<0.05$, $p<0.01$ and $p<0.005$).

3. Results

3.1. MELAS oxidative metabolism is impaired due to a severe complex I deficiency

The routine respiration i.e. the spontaneous mitochondrial O_2 cell consumption of the MELAS cybrid cells was significantly decreased by 39% compared to the parental cells (Figure 1A). The routine phosphorylating respiration dedicated to ATP production (difference between the routine and the respiration in the presence of oligomycin) was also reduced by more than 50% in mutant cells compared to parental cells (Figure 1B). Moreover, the maximal respiration of the mutant cells (i.e the respiration in the presence of FCCP, an uncoupler) was also reduced by more than 50%, compared to parental cells (Figure 1C), suggesting that the MELAS mutation impairs the respiratory chain routine and maximal capacity.

In addition, a severe complex I enzymatic activity deficiency with about 56% reduction was found in mutant cells compared to parental cells, while complex II and IV activities were not-significantly modified (Figure 1D). Moreover, complex I assembly was severely impacted by the MELAS mutation, with the presence of 400 kDa assembly intermediates (Figure 1E).

The metabolic consequences of the complex I deficiency were also evaluated in the culture medium of mutant cells compared to parental cells. Glucose consumption was significantly increased in mutant cells, and associated with a two fold increase of lactate production, leading to a reduction of pyruvate accumulation (Figure 1F), suggesting a metabolism highly reliant on the glycolytic pathway, and confirmed with a higher cytosolic NADH/NAD ratio in mutant cells (Figure 1F), indicator of the transformation of pyruvate into lactate.

3.2. Ketone bodies improve the mitochondrial respiration in mutant cells

Mutant cells were cultured in the presence of 5 mM of KB combined with a significant reduction of the glucose concentration to 0.5 g/L in the medium, compared to the regular high glucose (HG) medium (4.5 g/L). The KB doses were chosen in agreement with the concentrations seen in blood

of patients under ketogenic diet [22] and with concentrations previously used in *in vitro* studies [19].

Nevertheless, after 4 weeks of treatment, the routine respiration was significantly increased by more than 2 fold in KB MELAS treated cells compared to untreated cells (Figure 2A). Furthermore, the part of the routine respiration used to produce ATP was significantly increased by 76% under KB (Figure 2B), and similarly the maximal respiration was increased by more than two fold (Figure 2C). These results indicate that after 28 days, the KB treatment improved mitochondrial routine and maximal O_2 consumption of mutant cells.

3.3. Ketone bodies increase pyruvate metabolism and reduce the cytosolic NADH/NAD ratio in mutant cells

After 14 days of treatment, the glucose consumption, pyruvate and lactate production were not significantly changed compared to standard conditions (data not shown). However after 28 days of KB we found that the pyruvate consumption was significantly enhanced in mutant cells without increasing lactate level (Figure 2D), and without modifying the glucose consumption / lactate production ratio (Figure 2E), indicating that pyruvate was massively used by mitochondria. Moreover, these changes were associated with a significant reduction of the cytosolic NADH/NAD+ (Figure 2D). Taken together, these results suggest a restoration of the mitochondrial metabolism after 28 days of KB.

3.4. Ketone bodies reduce the mitochondrial NADH/NAD+ ratio in mutant cells

Within the mitochondrial compartment, the NADH is re-oxidized by the respiratory chain complex I into NAD+. The mitochondrial NADH/NAD+ ratio appears as a key component of mitochondrial metabolism. In order to understand the role of the NADH/NAD+ ratio in the restoration of the mitochondrial metabolism under KB, NAD^+ was added to permeabilized mutant

cells during oxygraphic measurements to stimulate the NADH formation through the Tri Carboxylic Acid (TCA) cycle. The O_2 consumption was first measured in standard High Glucose (HG) (Figure 3A1). The addition of NAD^+ had no effect on complex I mediated respiration but inhibited complex II and TCA cycle mediated respiration rates by 44% and 40% respectively compared to cells without NAD^+ addition. These results suggest an accumulation of NADH through the TCA cycle which retro-inhibits the TCA cycle linked respiration, ie TCA and complex II mediated respiration rates. However, after 28 days of KB, the complex I mediated respiration was significantly increased by more than 2 fold in response to the NAD^+ addition, without inhibiting complex II and TCA cycle-mediated respiration, suggesting the reoxidation of NADH through complex I (Figure 3A2).

To confirm our hypothesis, the mitochondrial NADH/NAD+ ratio was measured after 14 and 28 days of KB, revealing a 50% reduction at the end of the treatment, suggesting that the capability of NADH oxidation by complex I was increased in mutant cells (Figure 3B).

3.5. Ketone bodies restore the activity and assembly of complex I

In connection with the previous results, the enzymatic activity of complex I was measured in treated and untreated mutant cells. After 14 days of KB, no change was observed compared to untreated cells (not shown). However, the KB significantly increased complex I activity compared to untreated cells after 28 days, without significant effect on complexes II and IV (Figure 3C).

Complex I assembly of mutant cells was analysed using BN-PAGE. A major assembly intermediate at 400 kDa was seen under HG standard condition (Figure 4A). After 28 days of KB, assembly intermediates were drastically reduced and the proportion of complex I holoenzyme relative to the assembly intermediates was significantly increased compared to mutant cells grown in HG medium, from 72 to 98%, indicating a significant improvement of complex I assembly after long term exposure of KB (Figure 4A right panel).

3.6. Ketone bodies do not change the mutant load but increase the mtDNA copy number

The m.3243A>G mutation rate was measured on a regular basis, but no difference of the mutant load was seen after 28 days of KB exposure (HG: 98.6% +/- 0.1; KB: 98.7% +/- 0.3). However, after 28 days, KB significantly increased the mtDNA copy number by 44% in mutant cells (Figure 4B). Taken together, these results strongly suggest an increase of the absolute wild type mtDNA copy number in mutant cells due to KB treatment.

3.7. Ketone bodies promotes mitochondrial energetic metabolism in parental control cells

In order to determine the specific effects of the KB, the parental SH-SY5Y cells were exposed to KB treatment during 4 weeks of culture and different metabolites of cell metabolism were measured (Figure SI1A). After 28 days of treatment, the glucose consumption was not modified compared to standard culture condition but the pyruvate was massively used under KB treatment. The lactate production was significantly higher in KB treatment when compared to HG by approximately 2 fold. The cytosolic NADH/NAD ratio was not modified but the mitochondrial NADH/NAD+ ratio showed a 55% reduction after 28 days of KB, indicating that the NADH oxidation was also increased in parental cells (Figure SI1B).

The routine respiration of the control cells was not modified after 4 weeks of KB treatment (Figure SI1C). However, the part of this respiration used to produce ATP (i.e. the energetic efficiency) was increased by 57% after KB treatment (Figure SI1D). The mtDNA copy number was increased by 70% in parental cells (Figure SI1E).

4. Discussion

Ketogenic diet, consisting in a combination of high fat and low carbohydrate diet, appears as a promising therapy for mitochondrial disorders [23] but its beneficial effects and mechanisms of action are still unclear [8, 24]. The ketogenic diet and similar diets have been used in a large variety of neurological and metabolic disorders [25, 26]. Ketogenic diet has been used in several clinical studies to treat patients suffering from pharmaco-resistant epilepsies [13]. Seizure is part of the clinical phenotype in about 40% of children diagnosed with mitochondrial disease [15]. In a series of 14 children suffering from diverse mitochondrial diseases associated with intractable seizures, about 50% of patients became seizure-free after ketogenic diet but few of them did not show any benefit [27].

Several hypotheses have been raised regarding the mechanisms of neuroprotection of ketogenic diet. Ketogenic diet could have profound effects on neuronal plasticity and glutamate-mediated toxicity, reduces inflammation and improves bioenergetics by increasing ATP production and finally reduces ROS production, maybe through the induction of uncoupling proteins [26]. Although the effect of ketogenic diet is still not well understood, it is thought to mimic starvation when KB become the only fuel to fulfil the brain energetic requirements [28]. It was initially thought that ketogenic diet could promote the mitochondrial respiration through complex II activity and the oxidation of $FADH_2$, and therefore bypass the inactive complex I [16]. Hence, it was hypothesised that the resultant modification of the TCA cycle through the production of $FADH_2$ under ketogenic condition was the basis of the benefits of the regimen.

Among mitochondrial disorders, MELAS syndrome is considered as one of the most common maternally inherited diseases despite the fact that the pathogenicity of MELAS is still not well understood and we still have to come to a consensus for treating MELAS syndrome [9, 10].

Our study demonstrates a significant reduction of complex I activity combined with a reduction of complex IV albeit not significant as seen previously as an hallmark of MELAS pathophysiology [29]. Hence, Complex I defect appears as a prevalent feature in MELAS as reported before [4-6]. Complex I is the largest enzyme complex of the mitochondrial oxidative phosphorylation system with at least 44 subunits and is also described as the main site of ROS production within mitochondria [30]. The essential role of complex I is highlighted by the fact that inherited CI deficiency is the most common OXPHOS disorder, occurring in up to 30% of mitochondrial diseases [31].

We have shown that the improvement of the mitochondrial metabolism in MELAS cybrid cells after 4 weeks of KB exposure is almost exclusively mediated through the re-assembly and stabilisation of complex I, dramatically reducing the level of assembly intermediates, and consequently improving complex I activity and the routine phosphorylating respiration dedicated to ATP synthesis (summarized in Figure 5). In close connection, we have recently shown that the consequences of a misassembled complex I, and the accumulation of assembly intermediates were deleterious for the cell, by triggering ROS overproduction [32]. The level of complex I assembly intermediates was indeed strictly correlated with oxidative stress due to the presence of a still active N module of complex I which includes the catalytic domain responsible for NADH oxidation [32]. Hence, KB through the reassembly of complex I intermediates may act as an antioxidant process [26]. It was also shown that ketones prevented glutamate excitotoxicity in neocortical neurons by reducing oxidative stress following an enhancement of NADH oxidation and mitochondrial respiration [33]. In addition, increased longevity in mice was shown to be associated with an optimal assembly of mitochondrial complex I. indeed, biogenesis of the mitochondrial complex I is highly dependent on coordinate steps of membrane insertion of mtDNA encoded subunits with the matrix part of complex I. Indeed, a disruption of complex I assembly with an accumulation of assembled matrix arm subcomplexes of complex I reduced mitochondrial respiration while increasing ROS production [34].

A recent study has also shown that treatment of SH-SY5Y parental cells with decanoid acid (C10), a component of the medium chain triglyceride form of the ketogenic diet, increased citrate synthase and complex I activities, and the mitochondrial mass as well [35]. In our study, similar results were obtained when SH-SY5Y parental cells were exposed to KB during 4 weeks, promoting mitochondrial energetic metabolism with more ATP produced, through the mitochondrial respiration (Figure SI1), with a significant reduction of the mitochondrial NADH/NAD+ ratio and increasing the mtDNA copy number as well. However the lactate production was significantly higher in KB suggesting that the KB concentration used in our study was too high for the parental cells (Figure SI1).

The same C10 compound was also recently used in another study to expose fibroblast cells carrying nuclear-encoded mutations of complex I subunits responsible for Leigh syndrome during 6 days of cell culture [36]. As the main result, the supplementation using C10 had pleiotropic effects on mitochondrial metabolism of complex I mutant cells increasing citrate synthase activity in only 50% of patient cell lines and variable responses regarding ROS production or mitochondrial membrane potential in response to C10. Patient fibroblast cells carrying mutations in different genes encoding for key nuclear-encoded complex I subunits responded differently maybe in relation with the genetic defect. Mutant cells are more reliable on the glycolytic pathway due to a defective respiratory chain. Hence, reducing the level of glucose in the culture medium forces the cells to rely on mitochondrial respiration and alternative pathways to produce ATP. However, in the study of Kanabus et al it is interesting to note that the same glucose concentration (25mM) was kept during the treatment with C10 compared to untreated cells, which may interfere with the mitochondrial OXPHOS metabolism and may explain the cellular variability [36]. It has been suggested that glycolytic restriction is an important parameter to explain the anti-epileptic properties of the ketogenic diet [25]. In our study, to be as close as possible to the ketogenic regimen, which is also associated with low carbohydrates, KB

were combined with a significant reduction of glucose concentration in the culture medium (0.5g/l or 5 mM).

Interestingly enough, we have previously shown that the exposure of MELAS cells to low glucose alone (0.5 g/l) during 4 weeks shifted the heteroplasmy level from nearly 100% to 90% [18]. Shifting the level of heteroplasmy towards normal mtDNA and reducing the mutant load has become the goal of a large variety of invasive or non-invasive therapeutic approaches. This is consistent with data from cultured cells in which the mutant percentage of the m.3243A>G must exceed 90% before both protein synthesis and O_2 consumption decline [37]. Hence, a small decrease in mutant load or even a few percent increase of wild-type mtDNA copies in cells as seen in our study might be enough to rescue the phenotype if it passes under a pathogenic threshold of mtDNA mutation [38].

Moreover, KB has been also shown to reduce the percentage of deleted mtDNA molecules in cells carrying large-scale heteroplasmic deletions after only 5 days of treatment [19]. In our study, the KB did not change the percentage of mutant load which remained around 98.7%, suggesting that the improvement of mitochondrial complex I assembly and activity was not due to a change in heteroplasmy. The reason why the mutant load was stable using a similar dose of ketones compared to the study of Santra et al on cybrid cells carrying mtDNA deleted molecules is not well understood. Possible explanations are the different cell types that were used in both studies (143B cells *vs* neuronal cells) and maybe due to the mechanism of counter selecting deleted mtDNA molecules.

However, the mtDNA copy number of treated mutant cells under KB treatment was significantly higher compared to untreated cells indicating that the absolute amount of wild type mtDNA copy number was higher in mutant cells due to KB treatment. It has been shown that the mutant load is an important parameter but not sufficient to explain the variability of clinical phenotypes of mtDNA-related disorders. For instance, Leber's hereditary optic neuropathy (LHON), a maternally inherited optic atrophy due to mtDNA mutations is one of the most frequent mitochondrial diseases and is also characterized by incomplete penetrance. A clear difference

between asymptomatic LHON carriers and affected patients was seen in stimulating mtDNA replication highlighting the role of the mtDNA copy number as a possible explanation for the variable penetrance in LHON, and considered as a possible predictive genetic biomarker [39].

Finally, a 22-year old MELAS patient was treated with a ketogenic diet for about a year showing benefits in decreasing the frequency of stroke like episodes, although the mutant load or mtDNA copy number were not evaluated in this patient [16]. The next step is obviously to demonstrate the efficacy of the diet in MELAS using a randomized clinical trial. In such trial, it will be important to consider the type of KB given to the patients, as well as the duration of the effects of the regimen. Safety, tolerability and efficacy of KB has already been evaluated in different clinical trials for refractory epilepsy [27, 40-43] as well as in patients affected with mitochondrial disorders associated with intractable epilepsy [15].

5. CONCLUSIONS

In conclusion, we provide evidence about the interest of the KB supplementation in a neuronal model of MELAS cells. The level of CI assembly appears as a crucial parameter in alleviating complex I deficiency in mutant cells treated with KB. Hence, KB may appear as an alternative strategy to improve mitochondrial function, especially in MELAS, or more generally in mitochondrial disorders caused by complex I deficiency.

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Figure Legends

Figure 1. Comparison of mutant cybrid cells with parental control cells. (A) Oxygraphic measurements of mitochondrial routine respiration of control (ctrl) (black bars) and mutant cells (grey bars), n ≥ 4. (B) Oxygraphic measurements of the routine respiration used to produce ATP in control (black bars) and mutant cells (grey bars), n≥ 4. (C) Oxygraphic measurements of the maximal respiration in control (black bars) and mutant cells (grey bars), n≥ 4. (D) Complex I, II and IV/citrate synthase ratio of parental or control (ctrl) (black bars) and mutant cells (grey bars), n ≥ 4. (E) Blue-Native PAGE analysis of the complex I assembly in mutated and control cells. Complex I holoenzyme and assembly intermediates were visualized by enhanced chemiluminescence after primary antibody incubation (NDUFS2). Complex II holoenzyme is used as loading control. (F) Glucose consumption, pyruvate and lactate production and cytosolic NADH/NAD ratio of control (black bars) and mutant cells (grey bars), n ≥ 10. The data are presented as mean ± *sem* of the different experiments.

Figure 2. Effects of 28 days of KB on mitochondrial respiration and metabolism in mutant cells. (A) Oxygraphic measurements of mitochondrial routine respiration of mutant cells under High Glucose (HG) (black bars) or KB (white bars), n ≥ 3. (B) Oxygraphic measurements of the routine respiration used to produce ATP of mutant cells under HG (black bars) or KB (white bars), n ≥ 3. (C) Oxygraphic measurements of the maximal respiration of mutant cells under HG (black bars) or KB (white bars), n ≥ 3. (D) Glucose consumption, pyruvate and lactate production and cytosolic NADH/NAD ratio of mutant cells under HG (black bars) or KB (white bars) treatment, n ≥ 4. (E) Glucose consumption/lactate production ratio under HG (black bars) or KB (white bars), n ≥ 4. The data are presented as mean ± *sem* of the different experiments.

Figure 3. Mitochondrial NADH/NAD^+ ratio and respiratory chain complex measurements. (A) Mitochondrial O_2 consumption measured on permeabilized cells: Complex I and II and TCA cycle-dependent respiratory rates without (black bars) or with the addition of NAD^+ (white bars) under HG (A1) or KB (A2), $n \geq 5$. (B) Mitochondrial NADH/NAD ratio in KB treated cells at 14 and 28 days, $n \geq 7$. The mitochondrial NADH/NAD ratio was estimated through B-hydroxybutyrate/acetoacetate ratio. (C) Complex I, II and IV normalized to citrate synthase ratios after 28 days under HG (black bars) or KB (white bars), $n \geq 4$. The results are expressed as fold change relative to mean control cell values.

Figure 4. Assembly of complex I assembly level and mtDNA copy number in mutant cells.

(A) Blue-native PAGE analysis of respiratory chain complex I assembly after 28 days under HG or KB. The ratio of holoenzyme complexes and assembly intermediates were estimated as percentages, $n \geq 4$. (B) Mitochondrial mtDNA copy number in KB treated cells compared to HG medium, $n \geq 8$. The data are presented as mean + *sem* of experiments.

Figure 5. Schematic representation of the respiratory chain and the effects of KB in MELAS cells.

1: Complex I assembly is restored under KB. 2: Mitochondrial metabolism is induced with the consumption of pyruvate, with a reduction of the cytosolic NADH/NAD ratio; 3: Complex I activity is increased, oxidizing NADH, reducing the mitochondrial NADH/NAD ratio; 4 and 5: Increase of mitochondrial respiration dedicated to ATP synthesis.

Supplemental Figure SI1

Effect of the KB treatment on parental control cells. (A) Glucose consumption, pyruvate and lactate production and cytosolic NADH/NAD ratio of mutant cells under HG (black bars) or after

28 days of KB treatment (white bars) $n \geq 4$. The data are presented as mean $\pm$ sem of the different experiments. The asterisk (*) indicates significant differences ($p<0.05$).

(B) Mitochondrial NADH/NAD ratio in parental treated cells at 14 and 28 days, $n \geq 7$. The mitochondrial NADH/NAD ratio was estimated through B-hydroxybutyrate/acetoacetate ratio.

(C) O_2 Mitochondrial routine respiration of parental cells after 28 days under HG (black bars) or KB treatment (white bars) $n \geq 4$.. (D) Part of the routine respiration used to produce ATP of control cells after 28 days under HG (black bars) or KB treatment (white bars) $n \geq 4$.. (E) Mitochondrial mtDNA copy number in parental treated cells compared to HG medium, $n \geq 6$. The data are presented as mean + *sem* of experiments.

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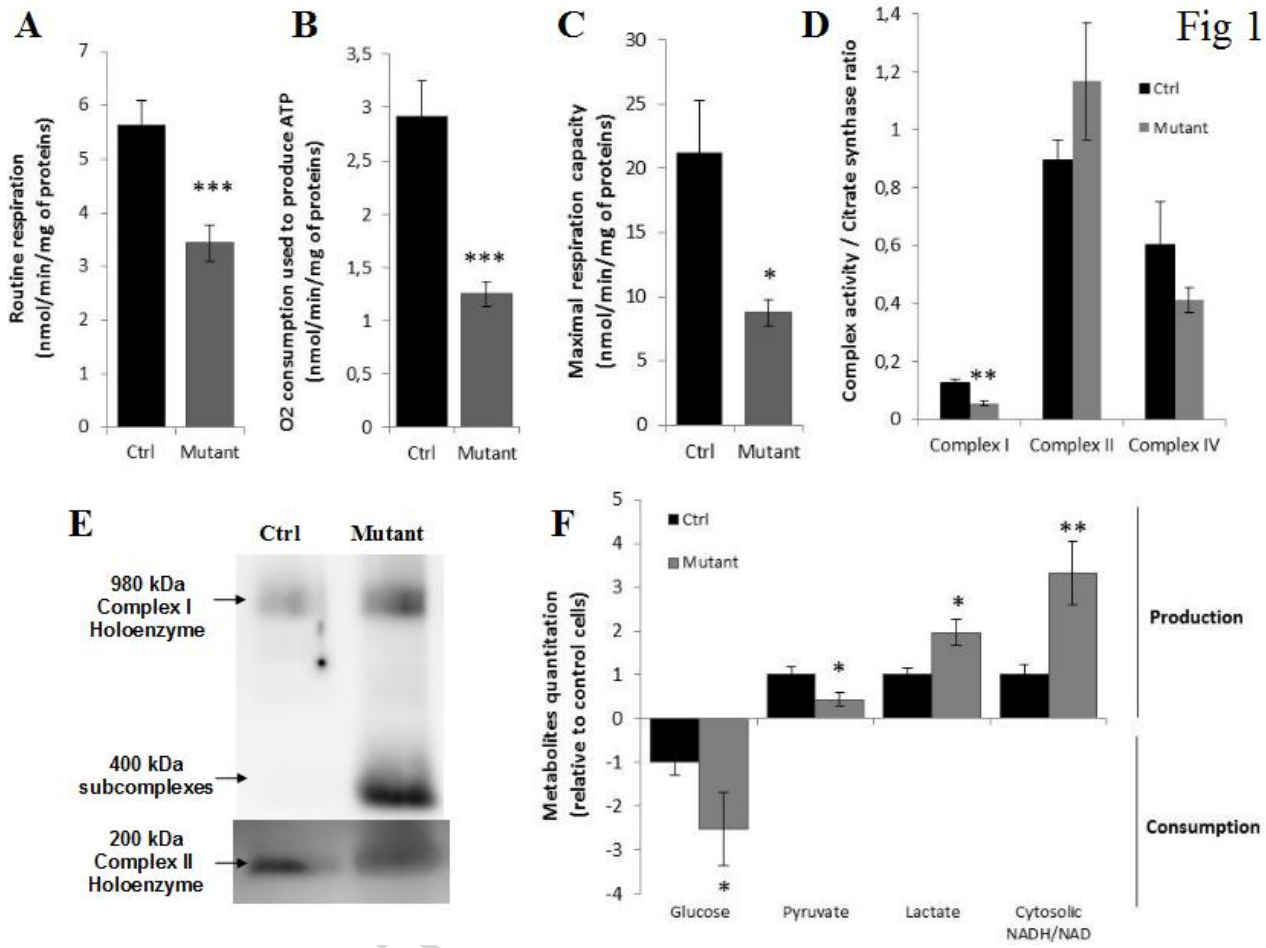
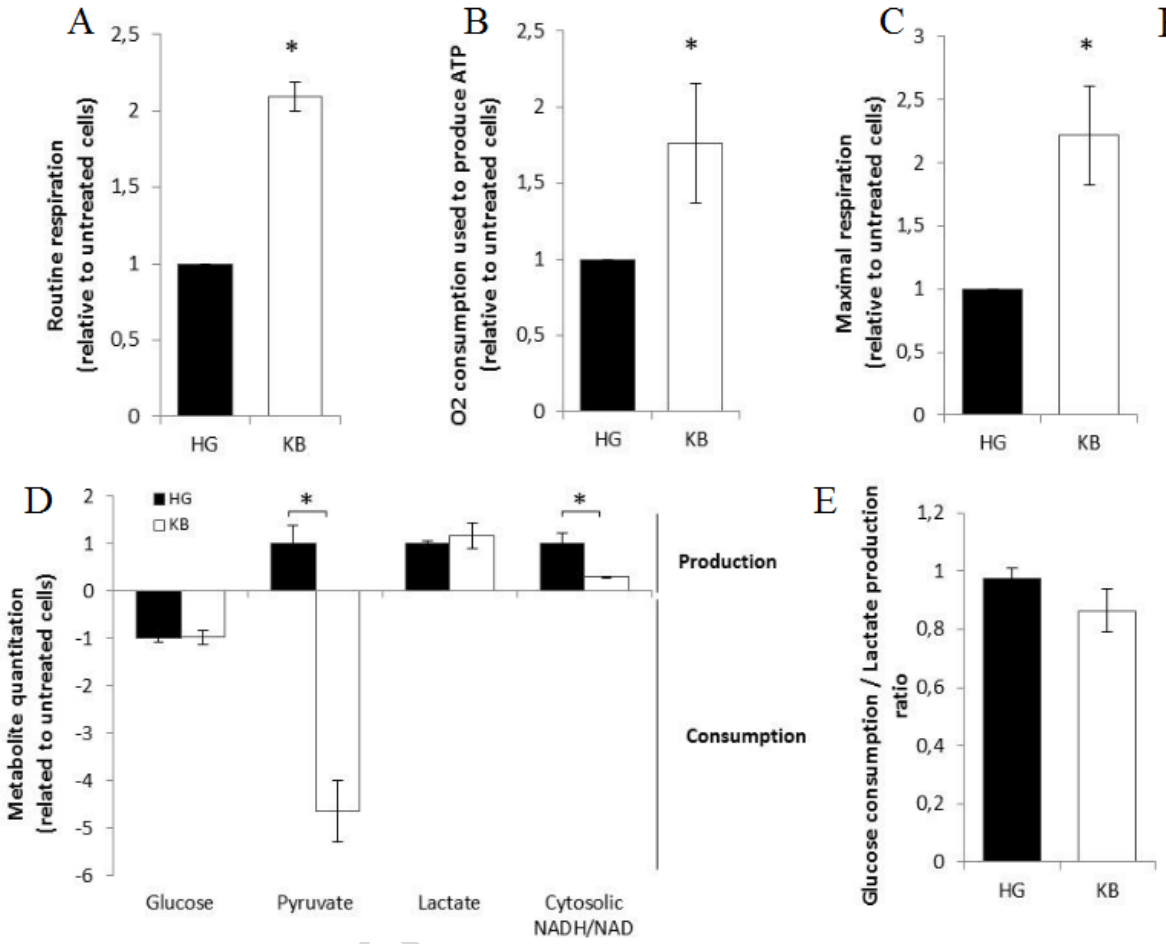


Fig 1

Fig 2



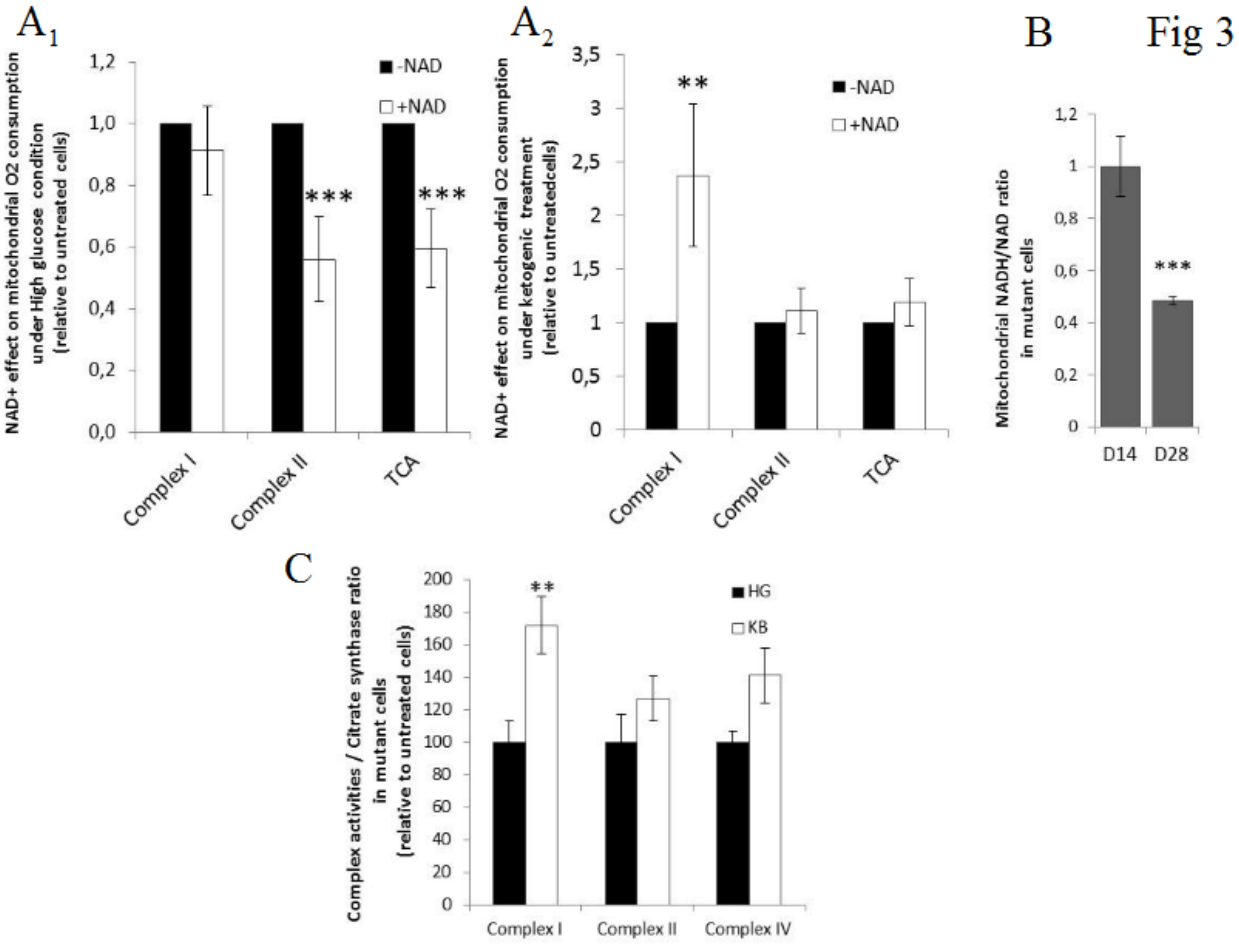


Fig 3

Fig 4

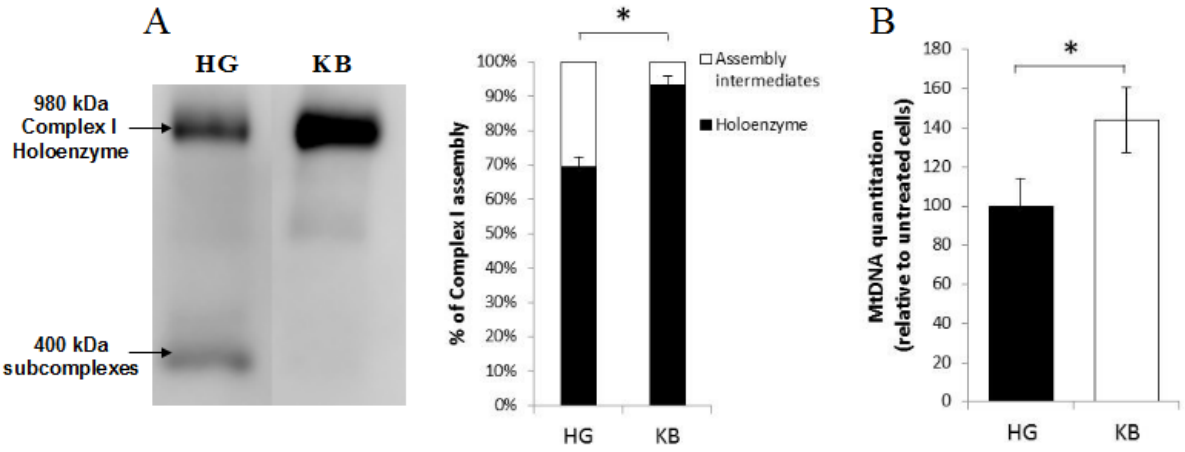
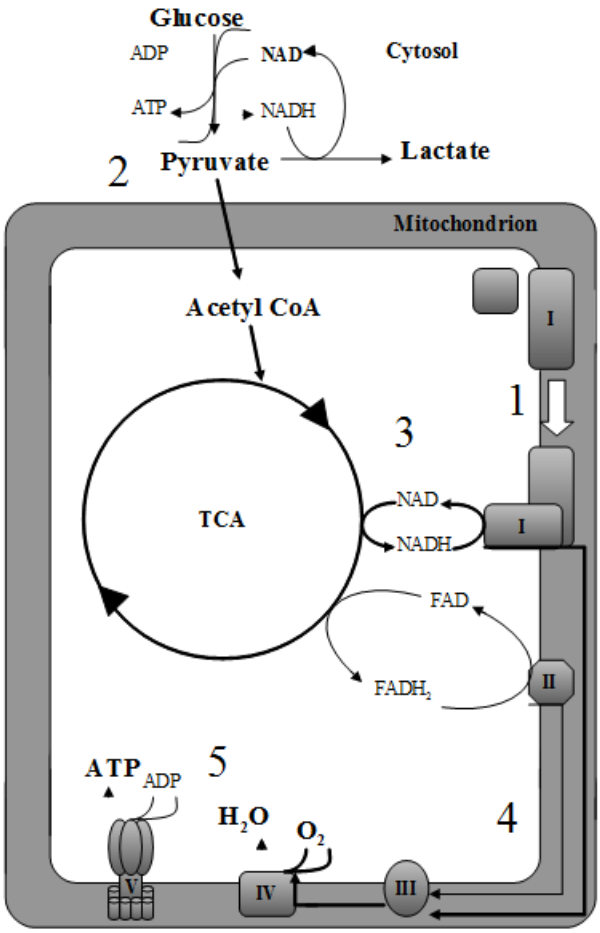


Fig 5



MELAS complex I deficient-cells

Highlights

- MELAS syndrome is associated with mitochondrial complex I deficiency and disassembly
- Ketone bodies improved complex I assembly and activity in MELAS neuronal cells
- Ketone bodies increases ATP synthesis and mtDNA copy number in treated mutant cells
- Ketone bodies may represent an alternative treatment for MELAS syndrome