Human NARP Mitochondrial Mutation Metabolism Corrected With α-Ketoglutarate/Aspartate

A Potential New Therapy

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Objective: To verify whether enhanced substrate-level phosphorylation increases viability and adenosine 5'-triphosphate (ATP) content of cells with neuropathy, ataxia, and retinitis pigmentosa/maternally inherited Leigh syndrome (NARP/MILS) mitochondrial DNA mutations and ATP synthase dysfunction.

Design: We used cell lines “poisoned” with oligomycin, the specific inhibitor of ATP synthase, and “natural” models, including transmitochondrial human cell lines (cybrids) harboring 2 different pathogenic mutations associated with the NARP/MILS phenotypes.

Main Outcome Measures: Cell survival, morphology, and ATP content.

Results: When normal human fibroblasts cultured in glucose-free medium were forced to increase energy consumption by exposure to the ionophore gramicidin or were energy challenged by oligomycin inhibition, their survival at 72 hours was 5%, but this increased to 70% when the medium was supplemented with α-ketoglutarate/aspartate to boost mitochondrial substrate-level phosphorylation. Homoplasmic cybrids harboring the 8993T→G NARP mutation were also protected from death (75% vs 15% survival at 72 hours) by the supplemented medium and their ATP content was similar to controls.

Conclusions: These results show that ATP synthase–deficient cells can be rescued by increasing mitochondrial substrate-level phosphorylation and suggest potential dietary or pharmacological therapeutic approaches based on the supplementation of α-ketoglutarate/aspartate to patients with impaired ATP synthase activity.

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Human mitochondrial diseases are associated with defects of oxidative phosphorylation (OXPHOS), which is the main energy-producing system in aerobic cells. Oxidative phosphorylation is catalyzed by the mitochondrial respiratory chain, which is composed of 4 multimeric complexes (I-IV) and the adenosine 5'-triphosphate (ATP) synthase. The energy generated by the electron transport chain (complexes I-IV) is harnessed as ATP by complex V (F1F0-ATPase or ATP synthase), a marvelous rotary engine. Altogether, the 5 OXPHOS complexes comprise nearly 90 different subunits, 13 of which are encoded by mitochondrial DNA (mtDNA), whereas all others are encoded by the nuclear genome, as are all factors needed for mtDNA replication and translation. Therefore, mutations of either mtDNA or nuclear DNA can cause mitochondrial diseases. The clinical features of these disorders are highly variable and can affect all tissues, although neurological symptoms and signs often predominate because of the high energy requirement of the nervous system (mitochondrial encephalomyopathies). Currently available treatment options for patients with mtDNA mutations are woefully inadequate and provide no more than temporary relief.

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Not surprisingly, the most severe consequence of OXPHOS diseases is energy deficiency, with partial or severe defects of ATP production, sort of cellular “brownouts” or “blackouts.” Thus, a metabolic approach to treat OXPHOS disorders appears logical, in the hope of increasing ATP supply to the cells by increasing substrate availability. Although this has not been extensively used in humans, experimental work in OXPHOS-deficient animal cells and yeast has shown that increases in substrate-level phosphorylation could generate enough ATP to correct energy defects caused by different mechanisms. This prompted us to grow human cells harboring pathogenic mtDNA mutations in α-ketoglutarate/aspartate-
We chose mutant cell lines carrying 2 distinct point mutations at the very same site of the gene encoding the ATPase 6 subunit of complex V, one of which (T8993G) impairs OXPHOS severely\(^7\) while the other (T8993C) affects OXPHOS only mildly.\(^8\) Both mutations are associated with neuropathy, ataxia, and retinitis pigmentosa/maternally inherited Leigh syndrome (NARP/MILS).\(^9,10\)

In addition, to verify whether cells made experimentally OXPHOS deficient by a different mechanism may also benefit from substrates supplementation, we studied the effects of this treatment in human fibroblasts exposed to oligomycin, the specific inhibitor of the mitochondrial ATP synthase. These 2 cell models ought to help us define whether OXPHOS-deficient cells can be rescued by exposure to \(\alpha\)-ketoglutarate/aspartate–enriched media.

**METHODS**

**CELLS AND PATIENTS**

We studied human fibroblasts and cybrid\(^11\) lines containing mtDNA derived from patients with NARP,\(^12\) as previously described.\(^13\) Control fibroblast cell lines were established from normal subjects with informed consent using standard techniques. Briefly, skin biopsy specimens were seeded in Dulbecco modified Eagle medium (DMEM) containing 4.5 g/L of glucose, 110 mg/L of pyruvate, and 4 mM glutamine supplemented with 20% fetal bovine serum (FBS), 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B and were incubated at 37°C in the presence of 5% carbon dioxide until fibroblasts grew out of the biopsy specimens. Cell lines were then expanded in complete medium supplemented with 15% FBS.

Growth curves and ATP levels of cells were obtained by seeding 2 \(\times\) 10\(^5\) to 5 \(\times\) 10\(^5\) cells in DMEM (Sigma D5030; Sigma-Aldrich, St Louis, Missouri) supplemented with 5 mM galactose, 110 mg/L of pyruvate, 0.8 mM glutamine, and 10% dialyzed FBS (DMEM-enriched medium) in the absence or in the presence of 3 mM \(\alpha\)-ketoglutarate + 5 mM aspartate. Dialyzed FBS was absent in the fibroblast culture medium. When specified, cells were cultured in complete medium with 40 ng/mL of gramicidin in the absence or in the presence of oligomycin (0.6 nM). In each experimental condition, equal numbers of cells were seeded in 9 dishes so that cell counts and ATP measurements could be obtained in triplicate for 3 days.

**CELL SURVIVAL**

Cell survival was assessed after growth in DMEM-enriched medium supplemented or not with substrates for 72 hours. Cells were washed with phosphate-buffered saline, trypsinized, collected, and, if necessary, diluted to 10\(^6\) cells/mL. The cells were then incubated in air at room temperature with an equal volume of trypan blue dye and counted by 3 independent investigators (mean [SD]). The number of viable cells was expressed as percentage of survival relative to the number of cells counted at the beginning of the experiment.

**MORPHOLOGICAL ANALYSIS**

Fibroblasts were plated in 60-mm Petri dishes and cultured for 72 hours. Petri dishes were washed once with phosphate-buffered saline and the cells were immediately visualized with an inverted microscope (Olympus IX50 [Olympus, Center Valley, Pennsylvania] equipped with a monochrome charge-coupled device camera). Multiple high-power (\(\times\) 20) images were acquired, and cells were scored as dead if they appeared smaller or shrunken and brighter, indicating release from the dishes.

**MEASUREMENT OF ATP CONTENT**

Adenosine 5’-triphosphate content was determined by measuring the light emitted during the oxidation of D-luciferin catalyzed by luciferase in the presence of ATP\(^14\) (ATP bioluminescent assay kit CLS II; Roche, Basel, Switzerland). Briefly, 30 µg

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**Figure 1.** Substrates (5 mM \(\alpha\)-ketoglutarate + 5 mM aspartate) rescue cell death in oligomycin-treated fibroblasts. Cells were incubated in Dulbecco modified Eagle medium–enriched medium (A), in the same medium plus 40 ng/mL of gramicidin (B), or in the same medium plus 40 ng/mL of gramicidin and 0.6 nM oligomycin (C), as detailed in the “Methods” section. Dotted lines denote presence of the substrates in the culture medium. Values are given as mean (SD).
of cell protein were suspended in 100mM potassium chloride, 10mM Tris/chloride, 5mM monobasic potassium phosphate, 1mM ethyleneglycoltetracetic acid, 3mM ethylenediaminetetraacetic acid, and 2mM magnesium chloride, pH 7.4, and ATP was extracted in dimethylsulphoxide. Adenosine 5’-triphosphate levels were quantified as nanomoles per milligram of protein and expressed as percentage of cellular ATP content at the beginning of the experiment (t=0).

Protein concentration was measured by the method of Lowry et al in the presence of 0.3% (weight to volume ratio) sodium deoxycholate. Bovine serum albumin was used as standard.

CHEMICALS

The following drugs and chemicals were used: tetramethylrhodamine methyl ester (Molecular Probes, Invitrogen, Carlsbad, California) and DMEM, FBS, penicillin, streptomycin, and amphotericin B (Gibco, Invitrogen). Oligomycin, trypan blue, α-ketoglutarate, aspartate, and all the buffer reagents were from Sigma-Aldrich.

STATISTICAL ANALYSIS

For all measurements, the values of all samples in different experimental conditions were averaged and the standard deviation of the mean was calculated. Where appropriate, we applied 1-way analysis of variance with multiple comparisons followed by Bonferroni post hoc test. Significance levels were set at $P < .01$.

RESULTS

RESCUE OF ENERGY-DEFICIENT RESTING FIBROBLASTS

By maintaining human resting fibroblasts up to 72 hours in glucose- and FBS-free medium, in which galactose (+ pyruvate) substituted for glucose, anaerobic glycolysis was almost abolished but cell viability was fully preserved even when cells were forced to increase energy consumption by exposure to the ionophore gramicidin (Figure 1A and B). Addition of 5mM α-ketoglutarate + 5mM aspartate (from now on referred to as “substrates”) to the medium did not affect significantly cell viability. In contrast, and as expected, adding to the medium low concentrations of oligomycin, the specific inhibitor of the F1F0-ATPase complex, caused a sharp decrease of viable fibroblasts, about 95% of which were dead at 72 hours (Figure 1C). This figure was reduced to less than 30% when substrates were added.

The protective effect of the substrates was confirmed morphologically: resting fibroblasts cultured for 72 hours in the galactose medium and forced to increase the rate of ATP synthesis by exposure to gramicidin (as explained earlier) had normal morphology (Figure 2A and B). However, when energy demand was increased and ATP synthase was inhibited by oligomycin (Figure 2C), the fibroblasts showed marked morphological changes, including shrinkage and detachment from the dish, typical features of cell death. In contrast, when the medium was supplemented with substrates, the fibroblasts retained normal morphology (Figure 2D, E, and F).

To verify whether the protective action of the substrates was due to their ability to supply ATP, we showed unequivocally that addition of the substrates to the medium conserved ATP (Figure 3). This was particularly evident in cells challenged by addition of both gramicidin, which stimulates the sodium potassium ATPase, and oligomycin (Figure 3C). Interestingly, the decrease of ATP preceded cell death, thus heralding cellular stress even when cell count and morphology were still normal (Figure 1C and Figure 3C).
EFFECT OF THE SUBSTRATES ON CYBRIDS HOMOPLASMIC FOR NARP T→G AND T→C MUTATIONS

Because addition of the substrates to the growing media was protective in fibroblasts with an artificially induced defect of ATP synthase, we investigated whether they could also prevent the death of homoplasmic cybrids carrying either 1 of 2 mtDNA mutations in the ATP6 gene. Substantial protection was observed only in the NARP 8993T→G cybrids (Figure 4A and B). Time course showed that, after 3 days, about 75% of the 8993T→G cybrids survived in substrates-enriched medium but only about 10% survived in the absence of substrates. Significantly, the decrease in ATP level of the 8993T→G cybrids paralleled cell death: residual ATP was nearly 10% after 3 days in the absence of substrates, whereas it remained higher than 80% when cells were grown in the presence of substrates (Figure 4C and D). In contrast, the growth curves of mutation-free (“wild-type”) and mutant homoplasmic NARP 8993T→C cybrids were similar (Figure 5A and B), indicating that the T→C point mutation does not affect significantly cell viability. Accordingly, the ATP content was also similar in wild-type and mutant cybrids (Figure 5C and D). Thus, both growth and ATP content of wild-type and NARP 8993T→C cybrids were unaffected by addition of the substrates to the culture medium.

COMMENT

Mitochondria are crucial for cell viability through several mechanisms, including ATP synthesis, ROS production, and regulation of programmed cell death (apoptosis). Besides their obvious pathogenic role in disorders due to defects of the respiratory chain (OXPHOS), mitochondrial dysfunction also contributes to aging and age-related pathologies, including cancer, cardiovascular disease, type 2 diabetes mellitus, and neurodegenerative disorders.19,20 Therapy for both primary mitochondrial diseases and late-onset neurodegenerative diseases is woefully inadequate, but there is a great fervor among researchers to identify strategies that might at least ameliorate symptoms. Advances in mitochondrial biology and in pharmacology have facilitated the design of drugs targeted to mitochondria.21,22 Although promising, this is still a young field, and there are concerns with “mitochondrial drugs” mainly because of our ignorance of the potential long-term toxic effects and our inability to regulate drug delivery to target tissues.23,24 A promising gene therapy approach is heteroplasmic shifting, aimed at lowering the mutant mtDNA below the pathogenic threshold by ingenious techniques, such as allotopic expression, but its applicability to humans appears remote.24

Cells with dysfunctional OXPHOS and energy brownouts have to maintain themselves on substrate-level phosphorylation, which cannot supply sufficient ATP when energy demand is high. In addition, since the main source of ATP produced through substrate-level phosphorylation is anaerobic glycolysis, a high glycolytic flux may induce lactic acidosis in neurons,25 which further impairs cell metabolism, hence, the need to pursue strategies other than potentiating glycolysis.

Recent articles suggest that “forcing” substrate-level phosphorylation to work overtime may be a viable approach to remedy the energy crisis due to OXPHOS impairment in yeast.4 These data bode well for application to patients. To optimize this strategy to human cells, we have chosen exogenous substrates capable of stimulating the Krebs cycle flux while at the same time remov-
ing the excess of reduced pyridine nucleotides (nicotinamide adenine dinucleotide [NADH]) in OXPHOS-deficient cells. To verify the validity of this approach in human cells in vitro, we have used both experimental models (in which cells are “poisoned” with a specific inhibitor) and “natural” models (transmitochondrial human cell lines harboring pathogenic mutations). As an experimental model, we chose resting fibroblasts because the most severely affected tissues in mitochondrial diseases are slowly proliferating postmitotic tissues, such as brain and muscle. Carrying out experiments under conditions of high energy demand in slowly proliferating fibroblasts better approximates the vulnerability of postmitotic tissues to mitochondrial dysfunction.

Both growth curves and ATP content of cells solely impaired in their ATP synthase capacity clearly showed the protecting effect of α-ketoglutarate/aspartate supplementation to the culture medium. This conclusion is bolstered by our observation that cells severely deficient in ATP synthase reacquired almost normal morphology following treatment (Figure 2). Since mitochondrial membrane potential (ΔΨm) is increased in cells with impaired ATP synthase, these cells are expected to have an increased NADH:nicotinamide adenine dinucleotide (NAD+) ratio, which would inhibit the α-ketoglutarate dehydrogenase reaction and possibly render the addition of the substrates useless. However, in our system, the α-ketoglutarate dehydrogenase reaction can still proceed because its NADH product is used to reduce oxaloacetate (provided as its precursor aspartate) to malate, which, in turn, may leave the mitochondrial matrix through the oxoglutarate carrier that exchanges it for α-ketoglutarate (Figure 6).

Notably, the substrates did not improve viability or ATP level of homoplasmic mutant mtDNA 8993 T→C cells. However, this is in keeping with our recent suggestion that the pathogenic mechanism of the T→C mutation may differ from that of the T→G mutation. We found that the T→C mutation causes only a marginal energy deficiency but a relatively high increase in ROS production, which might damage cell structure, induce the mitochondrial transition pore, and lead to apoptosis. A similar correlation between severity of the mutation and pathogenic mechanism (impaired ATP synthesis vs increased ROS production) has been documented in cultured cells harboring mutations in coenzyme Q10–synthesizing enzymes.

In conclusion, we have investigated the protective effect of exposure to α-ketoglutarate/aspartate in human cells with impaired OXPHOS due to mutations of the ATP6 gene. We obtained positive results in cells with severely inhibited ATP synthase but not in cells with mildly impaired ATP synthase. Therefore, we suggest that pa-
tients with some forms of ATP synthase deficiency might benefit from dietary or pharmacological approaches based on supplementation of α-ketoglutarate/aspartate, possibly associated with antioxidant therapy. We think that other patients with primary defects of energy production, including pyruvate dehydrogenase– and cytochrome c oxidase–related Leigh syndrome32,33 or defects of complex II,34 might also benefit from the α-ketoglutarate/aspartate treatment.

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