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THE MITOCHONDRIAL DNA MUTATION AT 8993 ASSOCIATED WITH NARP SLOWS THE RATE OF ATP SYNTHESIS IN ISOLATED LYMPHOBLAST MITOCHONDRIA

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SUMMARY: Mitochondria were prepared from three lymphoblast cell lines from patients with high percentage copy numbers of the human mtDNA 8993 mutation and compared to those prepared from related and non-related control cell lines. Rates of ATP synthesis with pyruvate/malate, succinate/rotenone, ascorbate/N'N'N'N' tetramethyl phenylene diamine were reduced to 67%, 58% and 54% of the control rates, repectively. The backward reaction measured as oligomycin sensitive ATPase was reduced to an average of 42% of that in controls. This mutation which changes a conserved leucine to an arginine in the putative membrane proton channel of mitochondrial ATPase effectively reduces the overall rate of oxidative phosphorylation.

The T to G point mutation at nucleotide 8993 of human mtDNA in the ATPase 6 gene has been associated with the human disease phenotype of neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) (1). In the 8993 mutation heteroplasmic variation in genotype i.e. the copy number of mutant versus normal mtDNA, has been found to be correlated with variation in phenotype. Thus high proportions of 8993 mutant mitochondria have been shown to be associated with Leigh disease (2,3) a neurodegenerative disease of the brainstem and basal ganglia while lower proportions give rise to the milder symptoms of ataxia and retinitis pigmentosa (1-3). Despite several studies on families affected by this mutation, no analyses of its effect on the process of oxidative phosphorylation has been published. In this study we set out to determine the effects of the 8993 mutation on the forward and reverse ATP synthetic reactions.

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Abbreviations: Complex I, reduced nicotinamide adenine dinucleotide-coenzyme Q reductase; Complex IV, cytochrome oxidase; Complex V, F_1F_oATPase.

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METHODS

Characterisation of Mutants. Lymphocyte cell lines of three Leigh disease probands were analysed for their content of the mtDNA 8993 mutation as described by Tatuch et al. (2). Also included in the study were two control cell lines from unrelated individuals, a control cell line from a first degree relative with 0% mutated mtDNA 8993, and cell lines from patients with complex 1 deficiency and cytochrome oxidase deficiency.

ATP Synthesis Assays. Lymphocyte mitochondria were prepared by the method of Bourgeron et al (4). 30 µg of isolated mitochondria were incubated in a 200 µl 0.25M sucrose, 2mM MOPS ph 7.4, 1mM EDTA, 5mM potassium phosphate, 1mM ADP solution (5). To assess the potency of various substrates with respect to ATP production, various substrates were added to this basic medium. Incubation was carried out for 1 hour at 37° C and 10 µl of 1.6 M perchloric acid was added to stop the reaction. The solution was cenrifuged to remove precipitated protein and the resulting extract assayed for ATP by enzyme fluorimetric methods (5).

ATPase Assays. 30 µg of isolated lymphocyte mitochondria were incubated in 200 µl of a hypotonic solution containing 50mM KCL, 20mM TRIS HCL ph 7.4, 2mM ATP, ±0.2 µg/ml oligomycin (6). The reaction was carried out for 1 hour at 37° C and 10 µl of 1.6 M perchloric acid was added to stop the reaction. Oligomycin-sensitive complex V ATPase activity was determined by assaying for inorganic phosphate using standard spectrophotometric methods (7).

RESULTS

ATP production was assessed in lymphocyte isolated mitochondrial preparations. In the control cell strains ATP production could be adequately stimulated by the addition of either 5mM pyruvate plus 0.1mM L-malate, 5mM succinate plus 1µM rotenone, or 2mM ascorbate plus 0.1mM N'N'N'N' tetramethyl phenylene diamine (TMPD) (Table 1). In patient 3563 (known to be complex I deficient) ATP synthesis was compromised with pyruvate plus malate as substrate but was adequate for either succinate plus rotenone or ascorbate plus TMPD. In patient 3945 (complex IV deficient) ATP synthesis was compromised with any of the three substrates. In three patient cell lines having >95% 8993 mutant mitochondrial DNA, ATP synthesis was significantly lower than in controls for all substrates tested. The average values for the three cell lines tested for ATP synthesis compared to controls were 68% for NAD-linked substrates, 58% for succinate/rotenone and 54% for ascorbate TMPD.

Complex V ATPase capacity was measured as oligomycin-sensitive ATPase activity in isolated lymphocyte mitochondria. Table 1 also shows that 8993 mutant cell lines have significantly lower ATPase activity than the controls with an average value for the three cell lines of 42%. Patient cell lines 3945 & 3563 (Complex I and IV deficient) showed normal activity of the oligomycin sensitive ATPase.

DISCUSSION

Mutations in mitochondrial DNA 8993 which are known to cause disease in affected family members appear to do so in a dose dependent fashion. Thus family members

Table 1. **ATP production and ATPase activity in isolated lymphoblast mitochondria**

Cell strain #	% 8993 mutant MtDNA	Production of ATP: No substrate	Pyruvate/Malate	Succinate/Rotenone	Ascorbate/TMPD	Oligomycin Sensitive ATPase Activity
		nmol/hour per mg protein				*nmol/min per mg protein*
Control 3781	0%	21±6(5)	1460±177 (5)	1218±143 (5)	867±87 (5)	25.5±1.6 (6)
Control 4189	0%	26±2(4)	1369± 33 (4)	1179± 44(4)	901±68 (4)	18.2 ±2.5 (5)
Patient 3969	>95%	25±4 (5)	951± 91 (5)	694± 82 (5)	432±62 (5)	7.6 ±0.6 (5)
Patient 4904	>95%	20±3 (5)	971±119 (5)	741± 44 (5)	443±42 (5)	11.9 ±1.5 (3)
Patient 3621	>95%	25±5 (5)	967± 76 (5)	652± 85 (5)	476±57 (5)	7.4 ±1.8 (3)
Patient 3645 (maternal nephew of 3621)	0%	26±2 (4)	1512± 53 (4)	1173± 103 (4)	740± 99 (4)	20.2 ±2.8 (5)
Patient 3945 (Complex IV)	0%	22±2 (3)	760± 71 (3)	566± 31 (3)	414± 50 (3)	18.2 ±0.5 (3)
Patient 3563 (Complex I)	0%	28±3 (4)	944± 85 (4)	1345± 44 (4)	764± 37 (4)	24.4 ±3.5 (3)

Values are given for the mean ±SEM, the number of determinations are given in parentheses. See text and methods for details. Activities were monitored in two control cell lines, one related control, three patients with >95% of the mt8993 mutation, one patient with cytochrome oxidase deficiency with Leigh disease and one patient with NADH-CoQ reductase deficiency with Leigh disease.

with low copy numbers of the mutant mtDNA in their mitochondria may be asymptomatic or may only develop retinitis pigmentosa in later life. In family members with high copy numbers of mutant mtDNA with more severe disease the extent of impairment experienced by the mitochondrial ATP synthetase is not known. In 3 individuals displaying >95% abnormal 8993 mtDNA the synthesis of ATP proceeded at 67% of the control rate with NAD-linked substrates and at a rate between 52% and 62% for succinate/rotenone and ascorbate/TMPD. Allowing for some contribution of the substrate level phosphorylation to the rate with NAD-linked substrates, this firmly shows that rates of oxidative phosphorylation were diminished in these mitochondria by close to one half. Decreases of this order of magnitude are also seen in the backwards reaction as monitored by the oligomycin sensitive ATPase. This implies that the ATP synthetic mechanism is constrained to work at a slower rate in utilizing the energy of the proton gradient generated by the respiratory chain. The impairment is also such that a comparable reduction in ATP synthesis is seen with that experienced in complex IV deficiency. It is not surprising then that both defects lead to an almost identical course of neurodegeneration (2,5).

The F_0F_1-ATPase catalyzes the terminal step in oxidative phosphorylation and photophosphorylation and is located in mitochondrial, chloroplast, and bacterial membranes. This enzyme is comprised of two major units, the hydrophilic extramembranous F_1 catalytic sector and the membrane bound F_0 which forms the proton pore (8,9,10). The F_0 of bacterial membranes is made up of three subunits a, b

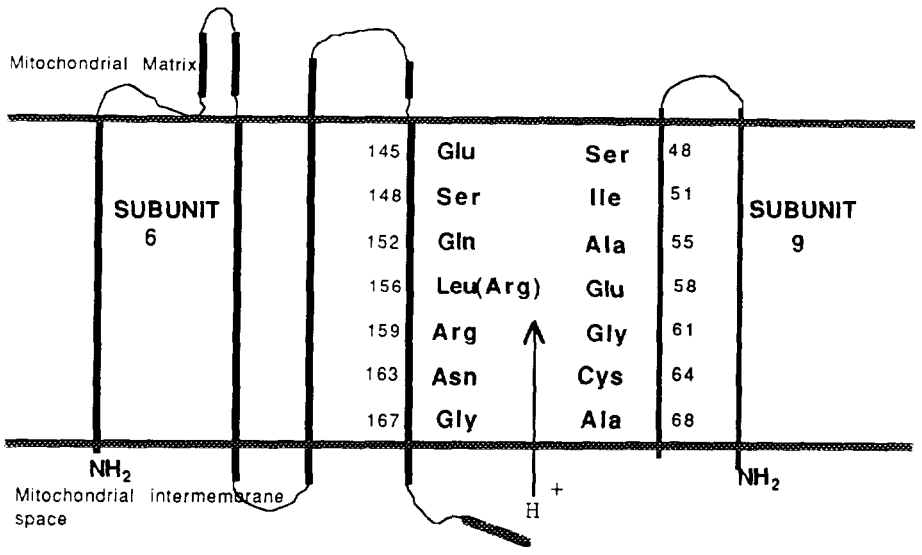

Figure 1. **The arrangement of the ATPase subunits 6 and 9 in the formation of the F_o ATPase proton channel.** Four amphipathic helices are shown for subunit 6, the amino acids facing into the protein pore in the human subunit are numbered. The second transmembrane helix of subunit 9 is shown in a similar fashion with numbered amino acid residues. The alteration is shown in brackets, Leu to (Arg) caused by the 8993 mutation.

and c and while the general membrane topology of subunits b and c is well accepted the structure of the a subunit is controversial, there being anywhere from 4 to 8 transmembrane helices (11-14). Bovine mitochondrial F_O is composed of at least three genuine transmembrane components: subunit a (subunit 6) and b (subunit 8) are encoded in mitochondrial DNA while the equivalent of protein c (subunit 9) is nuclear encoded (15). The 8993 mutation changes a highly conserved leucine 156 to an arginine in the fourth transmembrane helix of subunit 6 (Fig. 1). Modelling of the F_O proton channel shows that this has the effect of placing a positive charge in the immediate vicinity of glutamate residue on subunit 9 that is absolutely essential for the conservation of the energy of the electrochemical gradient in oxidative phosphorylation (2,11). Site directed mutagenesis of the equivalent leucine 207 in E. coli ATPase 6 (c subunit) to a tyrosine was effective in decreasing the growth yield on 10mM glucose minimal media (16) showing that the identity of this residue is important. Alternatively the arginine 159 of ATPase 6 which is sitting across from the crucial glutamate may form a salt bridge with the glutamate under normal circumstances but in the mutant the bridge may alternate between R159 and R156 thereby cutting the effective rate of protonation of the glutamate by 50%. The result of this imposition is a 50% reduction in the effective rate of oxidative phosphorylation.

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