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Mitochondrial ATP-Synthase Deficiency in a Child with 3-Methylglutaconic Aciduria

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ABSTRACT. We report the finding of mitochondrial ATP-synthase deficiency in a child with persistent 3-methylglutaconic aciduria. The child presented in the neonatal period with severe lactic acidosis, which was controlled by $NaHCO_3$ and glucose infusions. During the 1st y of life, there were several episodes of lactic acidosis precipitated by infections or prolonged intervals between meals. The excretion of lactate in urine was variable, but there was a persistent high excretion of 3-methylglutaconic acid. The activity of 3-methylglutaconyl-CoA hydratase in fibroblasts was normal. The child had a hypertrophic cardiomyopathy and magnetic resonance images revealed hypoplasia of corpus callosum. The gross motor and mental development was retarded, but there were no other neurologic signs. Investigation of muscle mitochondrial function at 1 y of age revealed a severe mitochondrial ATP-synthase deficiency (oligomycin-sensitive, dinitrophenol-stimulated Mg^{2+} ATPase activity: 27 nmol × min^{-1} × (mg protein)$^{-1}$, control range 223–673 nmol × min^{-1} × (mg protein)$^{-1}$. The mitochondrial respiratory rate was low and tightly coupled. The respiratory rate was normalized by the addition of an uncoupler. Low Mg^{2+} ATPase activity was also demonstrated by histochemical methods. Morphologic examination revealed ultrastructural abnormalities of mitochondria. There was no deletion of mitochondrial DNA. The sequences of the ATP synthase subunit genes of mitochondrial DNA were in accordance with published normal sequences. (*Pediatr Res* 32: 731–735, 1992)

Abbreviations

MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes
MERRF, myoclonus epilepsy with ragged-red fibers
mtDNA, mitochondrial DNA
nt, nucleotide
PCR, polymerase chain reaction

Increased excretion of 3-methylglutaconic acid was reported in two siblings with choreoathetosis, spastic paraparesis, dementia, and optic atrophy (1). Increased excretion of 3-methylglutaconic acid, which is an intermediate in the leucine catabolic pathway, has since been reported in more than 40 cases (2). In 1986, 3-methylglutaconyl-CoA hydratase (EC 4.2.1.18) deficiency (3) was found in two siblings with this organic aciduria, and recently one additional patient with this enzymatic defect was reported (4). However, in most cases with 3-methylglutaconic aciduria, no enzymatic defect has been identified. In 1983, Barth *et al.* (5) reported a large family with X-linked cardioskeletal myopathy, neutropenia, and abnormal mitochondria. Recently Kelley *et al.* (6) reported that a moderately increased excretion of 3-methylglutaconic acid was found in this disorder which they called Barth syndrome. The locus for this disorder has been mapped to Xq28 (7), but the primary defect has not been identified. Other cases with 3-methylglutaconic aciduria and severe neurologic impairment, some with lactic acidosis and/or cardiac involvement, have been reported, but no enzymatic defect has been identified (2, 8, 9).

Mitochondrial dysfunction is a cause of lactic acidosis in children, and cardiomyopathy is one of the manifestations of mitochondrial disorders (10, 11). Deficiencies of NADH: ubiquinone reductase (EC 1.6.5.3) (12–16), ubiquinol:cytochrome *c* reductase (EC 1.10.2.2) (17), and cytochrome *c* oxidase (EC 1.9.3.1) (18–20) have all been reported to be associated with cardiomyopathy in childhood. Cytochrome *c* oxidase deficiency alone (18–20) or combined with NADH:ubiquinone reductase deficiency (21–25) seems to be the most frequent finding. Mitochondrial ATP-synthase (EC 3.6.1.34) deficiency seems to be an extremely rare cause of oxidative phosphorylation impairment. It has only been reported in a 37-y-old woman with a hypertrophic cardiomyopathy who had suffered from a nonprogressive muscle weakness since childhood (26).

We report a child with 3-methylglutaconic aciduria, intermittent severe lactic acidosis and cardiomyopathy with severe mitochondrial ATP-synthase deficiency.

CASE REPORT

This girl was the third child of healthy nonconsanguineous parents, who were gypsies from Yugoslavia. The older children were healthy. The child was born at term. The delivery was normal. The birth weight was 2.2 kg. At 15 h she developed respiratory distress and a severe metabolic acidosis. At 2 d, investigation of organic acids in urine by gas chromatography/mass spectrometry revealed a severe lactic acidosis (55 mol/mol creatinine), slight ketoacidosis, and moderately increased excretion of 3-methylglutaric and 3-methylglutaconic acid (43 and 156 mmol/mol creatinine). The metabolic acidosis was controlled by $NaHCO_3$ and glucose infusions. Oral feeding was gradually introduced. At 1 mo, she had started to gain weight. The muscle tone was normal and she made good eye contact. She had no acidosis, but the lactate excretion was slightly increased. The excretion of 3-methylglutaric and 3-methylglutaconic acid persisted. At 4 mo, a hypertrophic cardiomyopathy was found. During her 1st y of life, she had several episodes of

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lactic acidosis that were precipitated by infection or prolonged intervals between meals. There was no hypoglycemia.

At 1 y of age, she was investigated for a mitochondrial disorder. Her weight was 5.9 kg. Muscle tone and mass were diminished. The gross motor development was at the level of 5–6 mo and fine motor and mental development at the level of 8–9 mo. The electroencephalography was normal, and there were no seizures or other neurologic signs. Magnetic resonance images showed hypoplasia of the splenium part of corpus callosum and slightly enlarged basal cisterns and ventricles. Fasting blood lactate concentration was increased to 3.4 mmol/L. The lactate:pyruvate ratio was increased to 28.6. The cerebrospinal fluid lactate concentration was 5.0 mmol/L, and the pyruvate concentration was 206 μmol/L. The cerebrospinal fluid protein concentration was slightly increased to 420 mg/L (reference interval 150–300). The total carnitine concentration in serum was high, 66 μmol/L (reference interval 23–60 μmol/L), and the acylated fraction was slightly increased to 16 μmol/L (reference interval <20% of the total carnitine concentration). Muscle carnitine concentration was normal, 16 μmol × g protein^{-1} (reference interval 7.4–26 μmol × g protein^{-1}). There were no signs of liver involvement, and serum aspartate aminotransferase and alanine aminotransferase were within the reference interval. Plasma prothrombin complex, ammonium ion, and amino acid concentrations were normal. Routine hematology, including neutrophil counts, were normal.

MATERIALS AND METHODS

A skeletal muscle specimen was obtained from the vastus lateralis muscle with the patient under general anesthesia. Morphologic examination, including enzyme histochemical and ultrastructural analysis of muscle tissue, was performed as described (10). Enzyme histochemical staining for mitochondrial Mg^{2+} ATPase was performed as described (27). Biochemical investigation of the respiratory chain function was done on isolated intact muscle mitochondria with polarographic measurements as described (10, 28) and in disrupted isolated mitochondria by spectrophotometric methods as described (10). Control biopsy specimens were from children investigated for suspected neuromuscular disease or undergoing orthopedic surgery. The diseases in these children were of nonmitochondrial origin, and there were no major morphologic or histochemical changes in the biopsy specimens (10). ATP-synthase activity was measured as the dinitrophenol-activated and oligomycin-sensitive ATPase activity in isolated muscle mitochondria according to Barth *et al.* (5).

DNA was isolated from muscle, blood, and fibroblasts from the patient, and mtDNA was analyzed by Southern blotting as previously described (29). The point mutation at nt 8344 (30) associated with MERRF syndrome was analyzed with PCR (31). The point mutation at nt 3243 associated with MELAS syndrome was analyzed with *Apa*I digestion and Southern blotting (32). The point mutation at nt 8993 associated with retinitis pigmentosa, and ataxia was analyzed with *Ava*I digestion and Southern blotting (33). Muscle DNA from the patient was used for mtDNA sequencing. The asymmetric primer method (34) was used to generate single-stranded DNA corresponding to the genes for ATP-synthase subunits 8 and 6. The single-stranded DNA was used either for direct sequencing as previously described (35) or for cyclic sequencing with the Taq DyeDeoxy Terminator Cycle Sequencing Kit and the 373A DNA Sequencer (Applied Biosystems Inc., Foster City, CA) under conditions recommended by the manufacturer. About 95% of the sequence was obtained by direct sequencing and the remaining 5% by cyclic sequencing. Primers corresponding to nt 8301–8320 (NG48), 8327–8346 (NG50), 8475–8494 (NG52), 8681–8700 (NG53), 8901–8920 (NG54), and 9106–9125 (NG55) of the heavy strand and nt 9250–9270 (NG49), 9228–9247 (NG51), 9030–9050 (NG56), 8835–8854 (NG57), 8585–8604 (NG58), and 8451–8470 (NG59) of the light strand of mtDNA were synthesized with a PCR Mate 391 DNA Synthesizer (Applied Biosystems Inc.). The primer pairs NG48/NG49, NG48/NG57, and NG53/NG49 were used for asymmetric PCR. The primers NG50–NG59 were used as sequencing primers. Numbering of mtDNA nucleotides was done according to the Cambridge human mtDNA sequence (36).

The activity of 3-methylglutaconyl-CoA hydratase in fibroblasts was measured by a coupled assay according to Gibson *et al.* (37). [5-^{14}C]-3-methylglutaconyl-CoA was generated by endogenous 3-methylcrotonyl-CoA carboxylase from 3-methylcrotonyl-CoA, ATP, and $NaH^{14}CO_3$. 3-Hydroxybutyrate dehydrogenase and NADH were included in the assay, and formed [1-^{14}C]-3-hydroxybutyrate was isolated by HPLC.

Organic acids were isolated from urine as described (38) and analyzed by gas chromatography/mass spectrometry on a TRIO-1 quadrupole mass spectrometer-data system (Fisons Instruments Nordic AB, Solna, Sweden) connected via an open split interface (Scientific Glass Engineering, Melbourne, Australia) to a Mega5160 gas chromatograph with on-column injector, CP-CF516 flow regulator, and AS550 autosampler (Fisons Instruments Nordic AB, Solna, Sweden). The gas-chromatographic capillary columns used were 30 m long, 0.33-mm inner diameter open tubular fused silica columns coated with 0.5 μm cross-linked SE-54 (Skandinaviska GeneTec AB, Kungsbacka, Sweden). Quantification of organic acids from reconstructed specific ion traces were done as described (38) using the LabBase software (Fisons Instruments Nordic AB, Solna, Sweden).

RESULTS

Initially, the severe neonatal lactic acidosis was the most impressive finding in this case. Slightly to moderately increased excretion of various other metabolites are often seen in critically ill infants, and initially the excretion of 3-methylglutaconic acid was regarded to be insignificant. It soon became evident that the increased excretion of 3-methylglutaconic acid was a constant finding, whereas there was a great variability in the amount of lactate excreted (Table 1).

3-Methylglutaconyl-CoA hydratase deficiency was excluded by measurement of 3-hydroxybutyrate production from 3-methylcrotonyl-CoA and ^{14}C-labeled bicarbonate in fibroblast extracts. The enzyme activity was measured at three different occasions. The mean activity in the patient was 102 pmol × min^{-1} × (mg protein^{-1}) (range 58–156). In 11 controls, the mean activity was 145 pmol × min^{-1} × (mg protein^{-1}) (range 83–348).

The intermittent lactic acidosis and the finding of a hypertrophic cardiomyopathy at 4 mo of age prompted an investigation for an oxidative phosphorylation disorder. At 1 y of age, a muscle biopsy was performed. The respiratory rate of isolated intact mitochondria was decreased in the presence of all substrates tested. The respiratory rate was normalized in the presence of an uncoupler (Table 2). The activities of the different parts of the respiratory chain measured in disrupted mitochondria were found to be normal (Table 3). The respiratory rate thus seemed to be limited by the coupling to ATP-synthesis. In accordance with this, a deficiency of ATP-synthase activity was found. The activity was measured as the DNP-stimulated oligomycin-sensitive Mg^{2+} ATPase activity in intact mitochondria (Table 4).

The histochemical investigation was in accordance with the biochemical measurements, and no detectable mitochondrial ATPase activity was found (Fig. 1), whereas normal cytochrome *c* oxidase and succinate dehydrogenase activities were found. Electron microscopy revealed abnormal mitochondria (Fig. 2).

The genes for ATP-synthase subunits 8 and 6 of mtDNA in muscle were sequenced and compared with the Cambridge human mtDNA sequence (36). We found a mutation that changes an adenine to guanine at nt 8860 and results in an amino acid replacement in subunit 6. This mutation has been reported in several patients with different neuromuscular disorders and also

Table 1. *Organic acids in urine*

Age	Lactate (mol/mol creatinine)	3-Methylglutaconate (mmol/mol creatinine)	3-Methylglutarate (mmol/mol creatinine)
2 d	55	156	43
3 wk	0.05	167	50
5 wk	4.8	135	41
2 mo	0.02	91	25
3.5 mo	0.03	120	20
7 mo	9.1	277	39
12 mo	<0.02	236	83
14 mo	<0.02	222	71
15 mo	0.03	284	68
Reference interval	<0.02	<20	<20

Table 2. *Oximetric measurements of respiratory rate in isolated muscle mitochondria*

	Respiratory rate [nmol O × min^{-1} × $(mg\ protein)^{-1}$]		
		Controls ($n = 10$)	
Substrate	Patient	Mean	Range
Pyruvate + malate	23	98	86–116
Succinate + rotenone	42	99	76–117
Succinate + rotenone + DNP*	122		
Ascorbate + TMPD†	159	251	207–302

* 2,4-Dinitrophenol, 125 μmol/L.
† N,N,N′,N′-tetramethyl-*p*-phenylenediamine, 500 μmol/L.

Table 3. *Respiratory chain enzyme activities in isolated muscle mitochondria*

	Enzyme activity		
		Controls ($n = 10$)	
	Patient	Mean	Range
NADH-ferricyanide reductase*	5900	6700	4500–8600
Succinate cytochrome *c* reductase*	340	300	160–400
Citrate synthase*	2700	2400	2000–3500
Cytochrome *c* oxidase†	13	11	6.1–15

* nmol × min^{-1} × $(mg\ protein)^{-1}$.
† Rate constant (k) min^{-1} × $(mg\ protein)^{-1}$.

Table 4. *ATP-synthase (Mg^{2+} ATPase) activity in isolated muscle mitochondria**

	ATP-synthase activity [nmol × min^{-1} × $(mg\ protein)^{-1}$]		
		Controls ($n = 10$)	
Additions	Patient	Mean ± SD	Range
+DNP−oligomycin	27	452 ± 179	223–673
−DNP−oligomycin	24	36 ± 21	0–72
+DNP+oligomycin	24	32 ± 16	0–57

* DNP, 2,4-dinitrophenol.

in healthy subjects. It probably represents an error in the Cambridge mtDNA sequence (30, 39). No other mutations were found. Southern blot analysis revealed no mtDNA deletions. The point mutations associated with MERRF, MELAS, and retinitis pigmentosa and ataxia syndromes were not found.

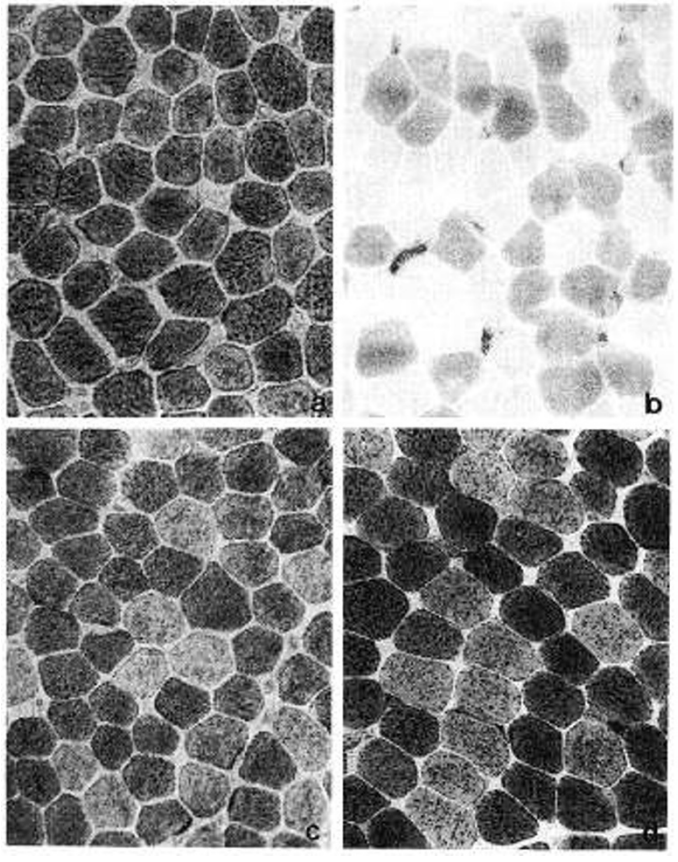

Fig. 1. Enzymehistochemical staining of cytochrome *c* oxidase (*a* and *c*) and Mg^{2+} ATPase (*b* and *d*) of skeletal muscle from the patient (*a* and *b*) and a control case (*c* and *d*). The particulate staining of mitochondria in the muscle fibers is absent in the patient after incubation for Mg^{2+} ATPase (*b*).

DISCUSSION

An isolated ATP-synthase deficiency has for the first time been identified in a child with hypertrophic cardiomyopathy, and a new enzymatic defect has been connected with 3-methylglutaconic aciduria. The family history of the patient was uninformative. We have made a thorough investigation of mtDNA, including sequencing of the mtDNA genes coding for the ATP-synthase subunits 6 and 8, and no deviations from known normal sequences were found. The congenital disorder of the girl is thus most probably an inherited autosomal recessive disorder, which may be due to a mutation in one of the probable 11 nuclear DNA-encoded subunits of ATP-synthase (40, 41).

ATP-synthase deficiency has only been reported in one previous case (26). This was a 37-y-old woman with a nonprogressive muscular weakness since childhood. There were no signs of

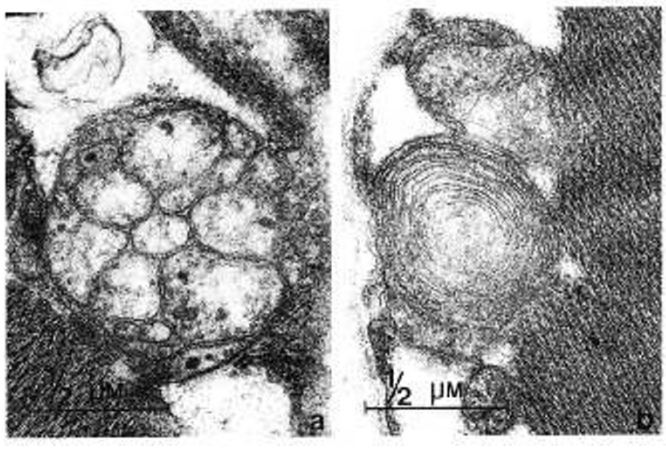

Fig. 2. Electron microscopy of abnormal mitochondria in the skeletal muscle showing abnormal arrangements of cristae.

encephalopathy. A point mutation of the ATP synthase subunit 6 gene of mitochondrial DNA has been found in a family with retinitis pigmentosa and ataxia (33). The ATP synthase activity in this family was not reported. It is thus not known if the phenotype of this family was linked to ATP synthase deficiency. The major problem in our case has been several episodes of severe lactic acidosis. These episodes were precipitated when intervals between meals were prolonged, and they were controlled by frequent feeding. The intolerance to fasting may indicate liver involvement, but there were no other findings that supported the possibility of liver disease. The increased lactate, pyruvate, and protein concentration in cerebrospinal fluid indicates involvement of the CNS. The child also had hypoplasia of corpus callosum. This has been reported in another case with 3-methylglutaconic aciduria (42). Malformations of the brain, including abnormalities of corpus callosum, are frequently found in pyruvate dehydrogenase deficiency (43) and may be caused by deficient energy metabolism. The clinical course in the previously reported case with ATP-synthase deficiency was rather benign (26). At present, the clinical course in our patient is uneventful. There has been no progress in the cardiac disease, and there have been no signs of a progressive encephalopathy. However, a future development of a progressive encephalopathy cannot be excluded, since there are signs of CNS involvement.

We cannot see an obvious connection between accumulation of 3-methylglutaconic acid and ATP-synthase deficiency. In our experience, 3-methylglutaconic aciduria is not a general finding in mitochondrial disorders. We have reported the results of extensive investigations of 50 children with suspected mitochondrial disorders (10). A mitochondrial disorder was verified in 20 of these patients. There was a record of increased excretion of 3-methylglutaconic acid in urine in two of these patients. At the time of the mitochondrial investigation, the excretion was normal in one of these patients and too modest to merit a diagnosis of an organic aciduria in the other patient. The first case had Kearns-Sayre syndrome. He was included in the study at 17 y of age at an advanced stage of the disease. A normal respiratory rate was found in the isolated mitochondrial preparation (10, 29). He died at 19 y of age in cardiorespiratory failure (44). The other case was a 6-y-old boy with a MERRF-like syndrome with generally deranged respiratory chain function. He had no signs of cardiac or liver involvement (10, 11). ATP synthase was not measured at the time, but histochemical analysis of a frozen muscle sample revealed normal mitochondrial ATPase activity. ATP-synthase activity was measured in the last 31 patients in the published series (10, 11). This series included patients with mtDNA deletions and the point mutations of MERRF and MELAS syndromes and patients with cardiomyopathy. In none of these patients was an ATP-synthase deficiency that would limit ATP synthesis found. Increased excretion of 3-methylglutaconic acid is thus neither a sign of impaired respiratory chain function in general nor a specific sign of ATP-synthase deficiency.

Mitochondrial cardiomyopathy is found in many patients with increased excretion of 3-methylglutaconic acid, *e.g.* in Barth syndrome (5), but 3-methylglutaconic aciduria is not a general finding in mitochondrial cardiomyopathy. 3-Methylglutaconic acid is an intermediate in the leucine-catabolic pathway. In patients with 3-methylglutaconic aciduria, which is not caused by 3-methylglutaconyl-CoA hydratase deficiency, the effect on the excretion of 3-methylglutaconate of leucine loads and leucine restriction has been reported. In some cases, moderately increased excretion has been found (1, 42, 45), but in most patients, *e.g.* those with Barth syndrome (6), the excretion seems to be independent of the dietary intake of leucine. Our patient was not given a leucine load or a leucine-restricted diet, but a remarkably constant level of excretion was found both in situations of metabolic stress and in situations of metabolic balance. This indicates that the excretion is independent of leucine catabolism. 3-Methylglutaconate is also involved in isoprenoid metabolism via the mevalonate shunt pathway (46). This pathway is proposed to account for significant metabolism of mevalonate to *n*-fatty acids and carbon dioxide. Increased flow or perturbation of this pathway, which may include metabolism of farnesyl pyrophosphate or other isoprenoid structures, may account for increased urinary excretion of 3-methylglutaconic acid. The most obvious connection between mitochondrial disorders and isoprenoid metabolism is ubiquinone (coenzyme Q), which is synthesized in the inner mitochondrial membrane by transfer of a polyprenylpyrophosphate to 4-hydroxybenzoate to form 3-decaprenyl-4-hydroxybenzoate (47), which then is hydroxylated, decarboxylated, and methylated to form ubiquinone. A perturbation of this synthesis could lead to increased degradation of, *e.g.*, polyprenylpyrophosphate and an increased production and excretion of 3-methylglutaconic acid.

Many patients with 3-methylglutaconic aciduria have signs and symptoms compatible with mitochondrial disorders. We have identified a specific mitochondrial disorder in a patient with 3-methylglutaconic aciduria. We do not think that 3-methylglutaconic aciduria is a specific sign of ATP-synthase deficiency, but rather a sign of a specific disturbance, which is caused by deficient oxidative phosphorylation.

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