

Increased reactive oxygen species (ROS) production and low catalase level in fibroblasts of a girl with MEGDEL association (Leigh syndrome, deafness, 3-methylglutaconic aciduria)

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Abstract

Association of 3-methylglutaconic aciduria (3-MGCA) with sensorineural deafness and Leigh-like encephalopathy (MEGDEL) was described as a very rare mitochondrial disorder without a known molecular background. We present clinical and biochemical characteristics of a 4.5-year-old girl with a similar association.

The clinical course of the disease was as follows: in the neonatal period transient adaptation troubles; at 4-5 mo failure to thrive and hypotonia; at 13 mo: extrapyramidal symptoms, sensorineural deafness, Leigh syndrome on MRI, pigmentary degeneration of retina, episodes of respiratory alkalosis, increased lactate in plasma, urine and brain, and increased excretion of 3-MGCA. Mitochondrial encephalopathy was suspected and muscle biopsy performed. Only mild lipid accumulation was found by muscle histopathology and histochemistry. Unspecific decrease of respiratory chain complexes was revealed by muscle homogenate spectrophotometry.

The in-gel activity assay in the patient's muscle confirmed a combined defect of OXPHOS, particularly indicating suppression of mitochondrial ATP synthase (complex V) activity. Measurements of functional mitochondrial bioenergetic parameters in the patient's fibroblasts revealed a decrease in the mitochondrial membrane potential and activity of the mitochondrial respiratory chain. At the same time, a significant increase of ROS production (cytosolic and mitochondrial superoxide and H₂O₂) with signs of protein damage (protein carbonylation), and decreased antioxidant defence (SOD1 and SOD2) were observed. Additionally, the catalase amount was surprisingly low in comparison with healthy control and other reference 3-MGCA cases (Barth syndrome).

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Conclusion: (1) the natural history of the disease in the presented patient confirms the existence of previously reported clinical phenotype of MEGDEL (2) antioxidant defence impairment due to abnormal catalase metabolism/transport may characterize an unknown basic defect which led to the development of MEGDEL association.

Key words: 3-methylglutaconic aciduria, MEGDEL association, Leigh syndrome, ROS production, antioxidant defence, catalase.

Introduction

Originally Leigh syndrome (LS, OMIM#256000) was a characteristic pathological gross and microscopic pattern of brain damage in autopsy study [13,25], now also detectable in vivo by computed tomography, magnetic resonance imaging and spectroscopy (MRI and MRS) [31]. In humans LS occurs almost exclusively in primary mitochondrial disorders, irrespective of the location of the defect in the process of oxidative phosphorylation, or of the type of the responsible gene mutation [14,16,28,30]. The changes are always of a similar location and nature, although they show varying degrees of severity and different age of appearance. The anatomical extent of involvement is progressive with time. LS is relatively frequent in the paediatric population in the country [18-21].

Non-specific 3-methylglutaconic aciduria (3-MGCA type IV, OMIM#250951) is a heterogeneous biochemical finding of unexplained pathogenesis [1,4,34,35]. This type of aciduria may occur in the course of mitochondrial diseases [9,15,23,33]. Careful analysis of the similarity of clinical presentation in a subgroup of children with 3-MGCA recently helped to identify a new gene, *TMEM70* [5]. Its mutations are responsible for mitochondrial cardiomyopathy and a deficit of mitochondrial ATP synthase (complex V) activity.

In 2006, another clinical subgroup of 3-MGCA type IV was identified [33]. These four children demonstrated a special association of symptoms, called MEGDEL association (methylglutaconic aciduria, deafness, Leigh syndrome). Only a few more similar cases can be found in the literature [6,9].

Retrospective analysis of our own material (over 300 patients with mitochondrial diseases including LS) showed that in the years 1995-2010, only a few could correspond to MEGDEL association. The aim of this study was to broaden knowledge about the pathogenesis of the defect on the basis of proteomic and functional studies of muscle and fibroblast mitochondria obtained from the girl with such an association.

Material and methods

Patient – case report

The 4.5-year-old girl, the daughter of healthy unrelated parents, was born on time (40 hbd) by Caesarean section, small for gestational age (weight 2200 g, Apgar score 10). Transient adaptation troubles and liver dysfunction were observed at 4-7 days of life (symptomatic hypoglycaemia 1.5 mmol/l, lactic acidemia 11.4, 13.4, 7.1 mmol/l, ATIII 41%, 61%, INR 2.08, 2.07). Neonatal test for phenylketonuria was false positive (normal serum Phe level and oral Phe loading excluded PKU). Blood amino acids and acylcarnitine profiles were normal with the exception of mild increase of methionine. Urinary profile of organic acids revealed increased excretion of 3-methylglutaconic, lactic, and p-hydroxypyruvic acids.

During early infancy her psychomotor development seemed normal; only mild hypotony was seen. Glucose concentration normalized, lactate concentration decreased (to 3.5, 3.2 mmol/l) ammonia level was in the normal range. A tendency to respiratory alkalosis was found in capillary blood (pH 7.47, pCO₂ 28.6 mmHg, pO₂ 55.6 mmHg, HCO₃ 20.5 mmol/l, BE -3.0 mmol/l, saturation 91.3%).

At the age of 7 mo she was admitted to a regional hospital because of failure to thrive (weight 5600 g) and enlarged liver (9 cm below costal margin). Laboratory data showed markedly increased transaminases (AspAT up to 1108 U/l), glucose level in the lower normal range, elevated plasma lactate (2.1-7.1 mmol/l), and compensated respiratory alkalosis (pH 7.429, pCO₂ 18.1 mmHg, pO₂ 90.3 mmHg, HCO₃ 13.4 mmol/l, BE -10.0 mmol/l). GC-MS analysis revealed mild 3-methylglutaconic aciduria, and increased excretion of lactic, p-hydroxypyruvic and 3-hydroxydicarboxylic acids.

At the age of 13 mo she did not sit and showed marked deficits in weight and growth (anthropometric measurements were from -2.0 SD to -3.0 SD). The liver was slightly enlarged. Sensorineural deafness (detected at 13 mo) was treated with a hearing aid device. Her emotional contact was preserved. Investi-

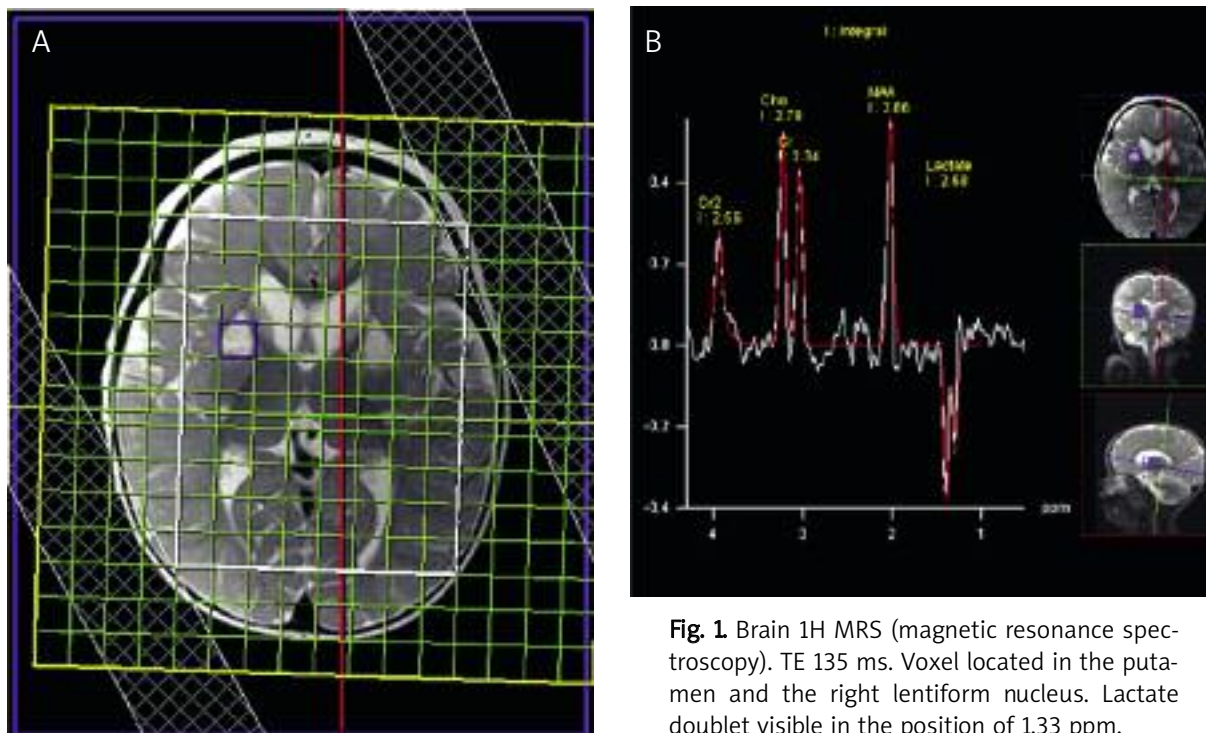


Fig. 1 Brain ^1H MRS (magnetic resonance spectroscopy). TE 135 ms. Voxel located in the putamen and the right lentiform nucleus. Lactate doublet visible in the position of 1.33 ppm.

gations revealed pigmentary degeneration of retina and Leigh syndrome features with lactate accumulation on brain magnetic resonance spectroscopy (Fig. 1). Cardiological examination was normal. Laboratory data showed increased plasma and cerebrospinal concentrations of lactate (4.7 mmol/l and 3.9 mmol/l) and alanine (621.1 $\mu\text{mol/l}$ and 56.6 $\mu\text{mol/l}$, respectively), liver dysfunction (AspAT/AlAT 140/102 U/l) and a switch from respiratory alkalosis to metabolic acidosis (pH 7.309, pCO_2 37.2 mmHg, HCO_3^- 18.4 mmol/l, BE -7.0 mmol/l).

Muscle biopsy performed at the age of 2 showed only a mild degree of lipid accumulation. Otherwise the biopsy was unremarkable. Histology and histochemistry revealed no features characteristic of mitochondrial disease/myopathy. Cytochrome oxidase activity was positive at the histochemical level. Spectrophotometric assay of muscle biopsy sample showed only unspecific changes of complex II/III activities (not shown). The search for known mitochondrial and nuclear gene mutations leading to LS was negative.

Mitochondrial disorder presenting as MEGDEL association of unknown biochemical and molecular background was diagnosed. Careful physical rehabilitation, stress avoidance and antioxidant preparations were administered.

From 17 months of life the girl demonstrated neurological deterioration, extrapyramidal signs and dystonic movements, as well as episodes of respiratory insufficiency.

At the age of 4 her status is relatively stabilized, she is fed through PEG, she breathes spontaneously, periodically receiving oxygen.

Histology, histochemistry and spectrophotometry

Histology, histochemistry and spectrophotometry of the muscle were performed according to the procedure described earlier [26,27].

Blue Native PAGE and in-gel activity assay

Samples for Blue Native electrophoresis from muscle biopsies were isolated as previously described [10,11]. Mitochondria were solubilized with 1.5 M 6-aminocaproic acid, 50 mM Bis-Tris pH 7.0, 1% dodecylmaltoside, combined with 0.5% Serva Blue G and separated on a 5-12% acrylamide gel. To visualize activity of individual mitochondrial respiratory chain complexes adequate parts of the Blue Native gel were incubated in the appropriate solutions as described [10,11].

Fibroblast cultures

Human skin fibroblasts were grown in Dulbecco modified Eagle's medium with glucose (4.5 g/l), 5 mM sodium pyruvate and 2 mM L-glutamine (Lonza), supplemented with 10% (v/v) fetal bovine serum (Gibco), and 1.2% antibiotic, antimycotic solution (Sigma Aldrich) in an atmosphere of 5% (v/v) carbon dioxide in air at 37°C. The cells were grown in 100 mm culture dishes. Three days before measurement of mitochondrial parameters cells were plated and grown in 24-well plates (Costar).

Estimation of antioxidant enzyme levels by Western blot technique

The level of antioxidant enzymes was investigated as previously described [12]. Cell lysates (35 µg protein) were separated electrophoretically in 10% SDS polyacrylamide gel and transferred onto PVDF membrane (BioRad). Membranes were blocked using 2% non-fat milk (Biorad) in TBS buffer containing 0.01% Tween 20 (Sigma Aldrich) for 1 h. Proteins were detected with anti-SOD1 rabbit polyclonal antibody (1 : 5000, Santa Cruz), anti-SOD2 goat polyclonal antibody (1 : 500, Santa Cruz), anti-catalase monoclonal antibody (1 : 1000, Santa Cruz) and anti-actin antibody (1 : 10 000, Abcam) followed by appropriate secondary HRP-conjugated antibodies (1 : 5000) (Santa Cruz).

Measurement of mitochondrial membrane potential ($mt\Delta\Psi$)

$mt\Delta\Psi$ was measured using 10 µM JC-1 as previously described [12].

Measurement of respiratory chain activity

The respiratory chain activity was measured in PBS containing 5 mM glucose and 6 µM resazurin as previously described [12].

Measurement of cytosolic superoxide ($cO_2^{\bullet-}$) production

$cO_2^{\bullet-}$ production was measured using 0.5 µM dihydroethidium (DHE) as previously described [12].

Measurement of mitochondrial superoxide ($mtO_2^{\bullet-}$) production

$mtO_2^{\bullet-}$ production was measured using 2.5 µM MitoSox as previously described [12].

Measurement of H_2O_2 production

H_2O_2 production was measured using 2 µM CM- H_2DCFDA as previously described [12].

Determination of protein modifications by oxygen free radicals

The level of oxidized proteins was estimated using the OxyBlot Protein Oxidation Detection Kit (Chemicon) as previously described [12].

Statistical analysis

Data obtained from the Tecan microplate reader were calculated using Microsoft™ Excel 2005 and analysed for significance by Student's *t*-test.

Results

Mitochondrial respiratory chain proteomic and bioenergetics parameters were investigated in the patient. First Blue Native electrophoresis and in-gel activity assay using muscle biopsy were performed. In-gel activity assay showed a significant decrease of complex V activity accompanied by a slight decrease of complexes II and IV activity in the female patient, which indicates a combined respiratory chain defect (Fig. 2A). Interestingly, similarly to the immunocytochemical results Western blot analysis showed no alterations in the amount of the studied respiratory chain subunits (Fig. 2B). This indicates that the mitochondrial alterations are connected with the defect in the enzymatic activity and not with the amount of the subunits of the mitochondrial respiratory chain complexes.

To study how these alterations affect basic mitochondrial parameters at a cellular level the fibroblast culture of the patient was investigated (Table I). In our recent paper [12] we reported that the measurement of mitochondrial membrane potential ($mt\Delta\Psi$) in such cells showed a significant decrease in the proton-motive force in mitochondria. Moreover, the activity of the respiratory chain measured using resazurin was reduced by ~25%.

Parameters describing intracellular oxidative stress were also assessed and found abnormal in comparison to the control fibroblasts (Table I, see also Figures for patient 2 [12]). The patient's fibroblasts demonstrated increased production of both cytosolic and mitochondrial superoxide. Moreover, the rate of H_2O_2 production was even doubled. The increased level of carbonylated proteins found in the patient's

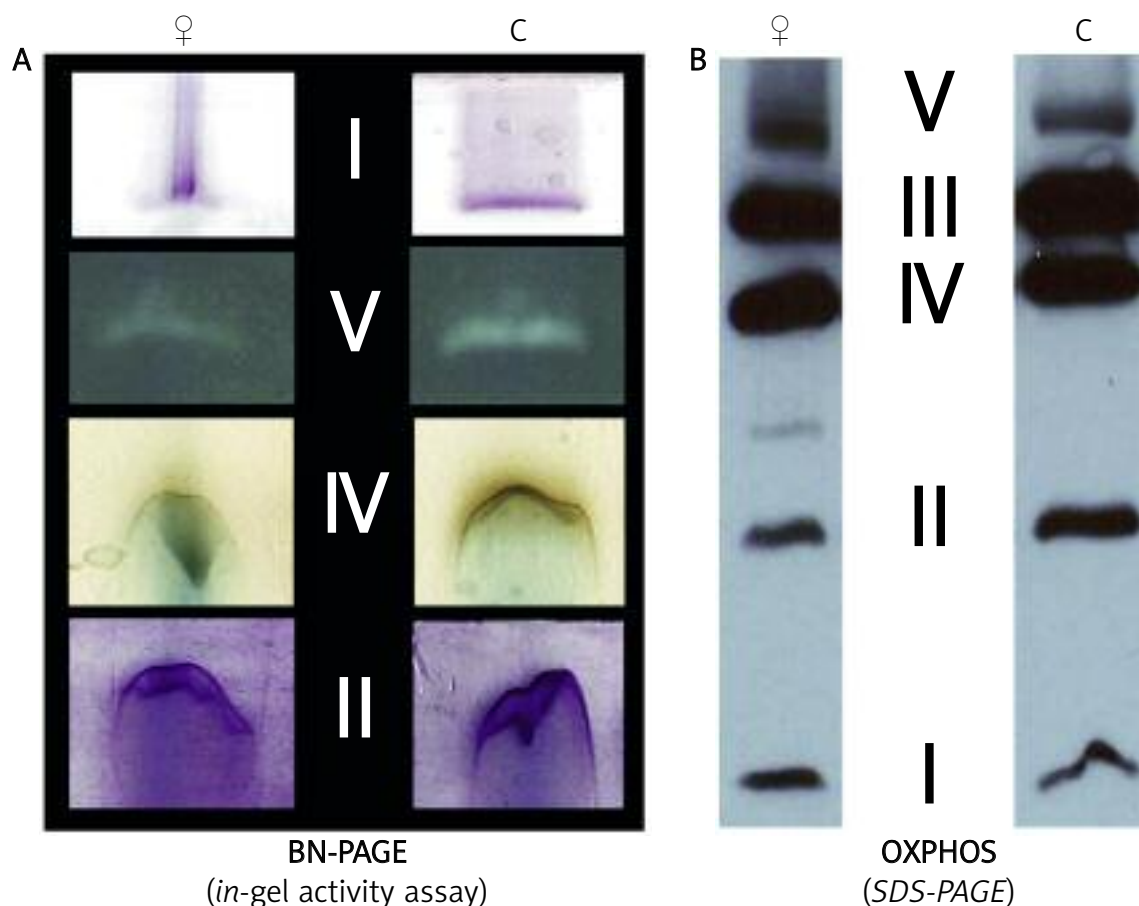


Fig. 2. Activity of mitochondrial respiratory chain complexes and the level of selected subunits of the respiratory chain complexes in muscle biopsy of a healthy individual and the patient. **A)** Activity of the mitochondrial respiratory chain complexes (I, V, IV and II) measured using in-gel activity assay performed after Blue Native Page from muscle biopsy of a healthy individual (C) and the patient (♀); **B)** Level of selected subunits of respiratory chain complexes (I, V, IV and II) from muscle biopsy of a healthy individual (C) and the patient (♀). A significant decrease of complex V activity accompanied by a slight decrease of complexes II and IV activity in the female patient together with no alterations in the amount of studied mitochondrial respiratory chain subunits is observed.

fibroblasts may also be connected with a decreased level of crucial antioxidant defence enzymes, including SOD2 and catalase.

The reduced catalase level in the patient's fibroblasts seemed especially interesting because it was not observed in the reference fibroblast lines of two brothers with Barth syndrome (BS). BS remains MEGDEL association by similar presence of the 3-MGCA urinary marker.

Discussion

The described patient was the first child among the dozens with Leigh syndrome with increased

excretion of 3-MGCA in the urine studied in our centre. The clinical picture included progressive encephalopathy, liver dysfunction, dystonia, pigmentary retinal degeneration, sensorineural deafness, increased lactate concentration in plasma, cerebrospinal fluid and urine, and 3-methylglutaconic aciduria. MEGDEL association described by Wartmann [33] was evident in the patient.

Diagnosis of mitochondrial disease in the patient (with 8 points on the scale of Nijmegen according to [17]) did not pose any difficulties despite the lack of explicit enzymatic and molecular background of genetic defect. Interestingly, episodes of hyperventi-

Table I. Mitochondrial function and ROS production and defence (as percentage of control value) in the patient's fibroblasts and reference BS fibroblasts

Parameter	Patient with MEGDEL association [%]	Barth syndrome brother 1 [%]	Barth syndrome brother 2 [%]
Mitochondrial membrane potential ($mt\Delta\Psi$)	75	33	33
Respiratory chain activity	70	92	89
Cytosolic superoxide production	200	75	211
Mitochondrial superoxide production	150	120	130
Rate of H_2O_2 production	250	63	56
Catalase level	8	330	270
SOD1 level	81	106	80
SOD2 level	57	42	44
Protein carbonylation	230	260	108

The presented values compiled and composed in to a presented table are based on our recent paper [12]

lation with hypocapnia and respiratory alkalosis were observed in the first phase of the illness, as we described in Leigh syndrome, independently from its molecular background [20,24,28]. According to our hypothesis, the reason for emergence of typical LS changes in the brain may be related to the subsequent increase in intracellular pH [22,24]. The current observation confirms that this phenomenon may also occur in MEGDEL syndrome.

Muscle histology and histochemistry of the patient revealed no significant pathology, and the activity of complex I and IV was in the normal range. Combined defect of respiratory chain function including decrease in the proton-motive force ($mt\Delta\Psi$) and low mitochondrial ATP synthase activity (with normal amount of complex V) was revealed by the expanded investigations in vitro and in vivo. Increased ROS production, decreased level of antioxidant defence, and remarkable protein carbonylation level were found [12]. There is increasing evidence that unbalanced ROS production and defence may contribute to the neuropathology [7].

Surprisingly, the amount of catalase in the patient was selectively low (8%) in comparison with the healthy control (100%) and the brothers with Barth syndrome (respectively 330% and 270%) [12]. The other measured parameters did not markedly differ between MEGDEL and BS fibroblasts. BS was applied as an example, referential for the other type of 3-MGCA pathology (Table I).

Catalase [8] is a known marker of peroxisome biosynthesis; and its lack in tissue subcellular structures is characteristic for Zellweger syndrome, a peroxisomal defect [3]. Moreover, increased excretion of 3-MGCA may be related to impairment of peroxisomal cholesterol biosynthesis through the sterol/isoprenoid metabolic pathway [2]. Low cholesterol levels observed in Barth syndrome (type II of 3-MGCA) was attributed to that mechanism.

Moreover, the clinical phenotype observed in our patient with MEGDEL association may be related to both mitochondrial and peroxisomal defects (deafness, pigmentary retinal degeneration, impaired liver function) [29]. Unfortunately, peroxisome function was not directly assessed in our MEGDEL case, and has not been reported in the literature.

Summary: In the expanded in vitro and in vivo investigations it has been revealed that the girl with Leigh syndrome, sensorineural deafness, pigmentary retinal degeneration and 3-methylglutaconic aciduria manifests a combined defect of respiratory chain function including decrease in the proton-motive force ($mt\Delta\Psi$) and low mitochondrial ATP synthase activity (with normal amount of complex V subunits). Also, as was described in our recent paper, increased ROS production, decreased level of antioxidant defence, especially very low catalase level, and remarkable protein carbonylation level were found. We speculate that an association of the specific clinical features with low catalase level/activity and with 3-methylglutaconic

acid accumulation may indicate the involvement of peroxisomal impairment in the MEGDEL syndrome, i.e. abnormalities of intracellular catalase transport.

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