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# A FATAL, SYSTEMIC MITOCHONDRIAL DISEASE WITH DECREASED MITOCHONDRIAL ENZYME ACTIVITIES, ABNORMAL ULTRASTRUCTURE OF THE MITOCHONDRIA AND DEFICIENCY OF HEAT SHOCK PROTEIN 60

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We report on a girl presenting with facial dysmorphic features and breathing difficulties upon birth. She was hypotonic, developed a metabolic acidosis, and died two days old of heart failure. Post-mortem examination revealed abnormalities of brain, lungs, heart and liver. In cultured skin fibroblasts activities of enzymes of oxidative phosphorylation, pyruvate metabolism, beta-oxidation and other mitochondrial (mt) metabolic pathways were markedly decreased. Activities of enzymes localized in the mt outer membrane or in other cell organelles were found to be normal. The mitochondria appeared swollen and were located mainly around the nucleus. Electron micrographs showed locally disintegrated mt inner membranes and large mt vacuoles. The amount of mt heat shock protein 60 (hsp60) was about one fifth of that in controls. We conclude that this mt disorder is most likely caused by defective synthesis and maintenance of mitochondria, possibly due to a defect in mt protein import or enzyme assembly resulting from deficiency of hsp60. 

Mt encephalomyopathies associated with lactic acidosis constitute a heterogeneous group of metabolic diseases with regard to the clinical manifestation as well as to the underlying biochemical defect (1). Clinical symptoms can vary from

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a life-threatening condition as a result of extreme hypotonia and severe acidosis shortly after birth to exercise intolerance at later age. Biochemical defects encompass deficiencies of the enzymes of oxidative phosphorylation, pyruvate metabolism and the citric acid cycle. In some patients defects in oxidative phosphorylation have been found to be associated with mutations of the mt DNA (2). The genetic basis of most of the other cases with apparently normal mt DNA is not known.

Besides single enzyme defects, combined deficiencies of mt enzymes have been described as well (1). The enzyme complexes affected nearly always comprised the pyruvate dehydrogenase complex and one or more enzymes of the respiratory chain. Here, we present a patient whose fibroblasts exhibited decreased activities of 10 mt enzymes out of the 11 measured. We document the further metabolic characterization of these fibroblasts, and the morphology and ultrastructure of the mitochondria. In addition, we report on experiments aimed at the identification of the basic defect of this mt disorder.

## CASE REPORT

The patient investigated was a girl, second child, born after an uneventful pregnancy. Family anamnesis: her parents were first cousins, the elder sister died three days after birth showing a clinical picture similar to that of our patient, the younger brother is healthy. After delivery the patient was in bad condition and had breathing difficulties. She had facial dysmorphic features, slight frontal bossing and hypoteleorism. There was much hair on the body. The child gradually developed a metabolic acidosis (pH 7.29). She had a high degree of hypotonia and a severe lactic acidosis of 5.5 mmol/liter. Organic acids in the urine only showed accumulation of lactate. Investigations for infections were negative. Cardiological examination showed persistent foetal circulation and dextrocardia. The child died two days after birth. Pathological examination revealed a situs ambiguus with the heart displaced to the right. The ductus arteriosus was still patent. Grossly, the lungs had a normal appearance, histologic examination showed reduced air spaces in the lung alveoli and thickened alveolar walls. The liver was enlarged, histologic examination revealed no abnormalities. Neuropathological examination showed acute hypoxic cerebral damage (pontosubicular neuron necrosis). In addition, there was a periventricular infarct, some days old, with astroglia response, which probably had occurred in utero. Striking microcystic changes were observed in the tegmental part of pons and medulla oblongata.

## MATERIALS AND METHODS

**Antisera.** Rabbit polyclonal antibodies to human and yeast hsp60 were raised as described previously (3,4). Antiserum raised against cytosolic hsp70 (bovine brain uncoating ATPase) was a generous gift from C. Wiegand and Dr. B.M. Jockusch, University of Bielefeld (Germany).

**Measurement of enzyme activities.** Lactate and pyruvate produced by fibroblasts upon administration of glucose were measured as described by Wijburg et al. (5). Activities of carnitine palmitoyltransferases I and II (6), cytochrome c oxidase (7),

dihydroxyacetone phosphate acyltransferase (8), fumarylacetoacetate hydrolase (9), beta-galactosidase (10), glucose-6-phosphate dehydrogenase (11), leucine decarboxylation (12), 2- ketoglutarate dehydrogenase (13), medium chain acyl-CoA dehydrogenase (14), $Mg^{2+}$ ATPase (15), propionyl-CoA carboxylase (16,17), pyruvate carboxylase (16), pyruvate dehydrogenase (13) and succinate cytochrome c reductase (7) were measured essentially according to the methods published in the references indicated. Activation of pyruvate dehydrogenase was achieved by preincubation with 1.7 mM $Ca^{2+}$ + 1.5 mM $Mg^{2+}$, and inactivation by preincubation with 10 mM ATP.

**Western blots and immunodetection of proteins.** Electrophoresis and western-blotting of cell extracts were performed according to standard procedures (18). Incubation with diluted antiserum was overnight. The antigen-antibody complexes were detected using the alkaline phosphatase-coupled IgG system (Boehringer).

**Fluorescence staining of mitochondria.** In vivo staining of mitochondria with the fluorescence dye 2-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (DASPMI) was performed as described previously (19).

**Electron microscopy.** Cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, supplemented with 1mM $CaCl_2$ and 1 mM $MgCl_2$ for 90 min at room temperature and prefixed in a 1:1 mixture of 1% $OsO_4$ and 2.5% $K_2Cs_2O_7$ in the same buffer for 60 min. After poststaining in 1% uranylacetate the samples were dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips CM10 electron microscope.

## RESULTS

**Fibroblast cultures, lactate-pyruvate production from glucose.**

The skin fibroblasts from the patient adhered readily to the surface of culture flasks and showed good viability over 25 passages. The growth rate was slightly lower than that of control fibroblasts. After starvation and subsequent administration of glucose the patient cells produced a high amount of lactate resulting in a lactate-pyruvate ratio of 210 as compared to 60-80 in controls.

**Mitochondrial and other cellular enzyme activities.**

The activities of 11 multimeric mt enzymes were determined, the results are shown in Table I. With the exception of carnitine palmitoyltransferase I activity, all mt enzyme activities were decreased in the patient cells. Activities of cytochrome c oxidase and succinate cytochrome c reductase (antimycin-resistant) were less than 10% of control values. Residual activities of pyruvate dehydrogenase, 2-ketoglutarate dehydrogenase and medium chain acyl-CoA dehydrogenase and of leucine decarboxylation were between 10-30% of the corresponding control values. Pyruvate carboxylase, propionyl-CoA carboxylase, $Mg^{2+}$ ATPase and carnitine palmitoyltransferase II were the least affected enzymes, but activities were clearly lower than those measured in control fibroblasts. In contrast, activities of peroxisomal (dihydroxyacetone phosphate acyltransferase), lysosomal (beta-

TABLE I ACTIVITIES OF MITOCHONDRIAL ENZYMES

| Activity (expressed in nmol substrate. $min^{-1}.mg^{-1}$) | Patient | Control ranges (in brackets the number of controls) |
|---|---|---|
| Pyruvate dehydrogenase ($1-^{14}C$-pyruvate) | | |
| . basal | 0.32 | 0.92; 1.22 |
| . after activation by $Ca^{2+}$ and $Mg^{2+}$ | 0.20 | 0.89; 1.24 |
| . after inactivation by ATP | 0.125 | 0.52; 0.71 |
| Pyruvate carboxylase | 0.061 | 0.17-0.85 (20) |
| Cytochrome c oxidase (cyt c $Fe^{2+}$) | 0.16 | 0.94-2.90 (9) |
| Succinate cyt c reductase (antimycin-sensitive) (cyt c $Fe^{3+}$) | 0.85 | 6.81-13.7 (6) |
| $Mg^{2+}$-ATPase (oligomycin-sensitive, uncoupler stimulated) | 8.70 | 13.33 |
| 2-Ketoglutarate dehydrogenase ($1-^{14}C$-2-ketoglutarate) | 1.90 | 6.30; 7.07 |
| Carnitine palmitoyltransferase I | 0.57 | 0.43-1.46 (6) |
| Carnitine palmitoyltransferase II | 0.30 | 0.54-0.75 (6) |
| Medium chain acyl-CoA dehydrogenase | 0.59 | 0.98-2.56 (15) |
| Propionyl-CoA carboxylase | 0.21 | 0.25-1.20 (20) |
| Leucine decarboxylation | 2.4 | 14.2; 16.4 |

galactosidase) and cytosolic (glucose-6-phosphate dehydrogenase and fumarylacetoacetate hydrolase) marker enzymes were within the activity ranges measured in control fibroblasts (Table II). These results demonstrate that the multiple decrease in enzyme activities was specific for the mitochondria whereas other cellular activities were normal.

**Mitochondrial morphology and ultrastructure.**

Mitochondria were visualized by staining with the fluorescence dye DASPMI, which is selectively taken up by the mitochondria due to their transmembrane potential. The efficient uptake of the dye by the patient mitochondria proves that in spite of the low activities of the respiratory chain enzymes a transmembrane potential was still generated. However, morphology and distribution of the patient mitochondria were clearly altered. Mitochondria in control cells were visible as rod-

TABLE II ACTIVITIES OF CYTOSOLIC, LYSOSOMAL AND PEROXISOMAL ENZYMES

| Activity (expressed in nmol substrate.min$^{-1}$.mg$^{-1}$) | Patient | Controls (in brackets the number of controls) |
|---|---|---|
| Glucose-6-phosphate dehydrogenase | 196 | 76 - 202 (11) |
| Fumarylacetoacetate hydrolase | 3.42 | 1.52 - 3.11 (8) |
| Beta-galactosidase | 14.8 | 7.5 - 20.8 (80) |
| Dihydroxyacetone phosphate acyl-transferase | 0.21 | 0.12 - 0.27 (6) |

like structures of equal diameter (Fig. 1A). The mitochondria of patient cells were swollen and irregularly shaped and were mainly localized around the nucleus (Fig. 1B).

Electron microscopic examination of the patient cells revealed that the mitochondria were large and predominantly clustered around the nucleus (Fig. 2). Remarkably, the mitochondria had locally disintegrated inner membranes. Different stages of membrane disintegration, from minor disarrangements of the inner

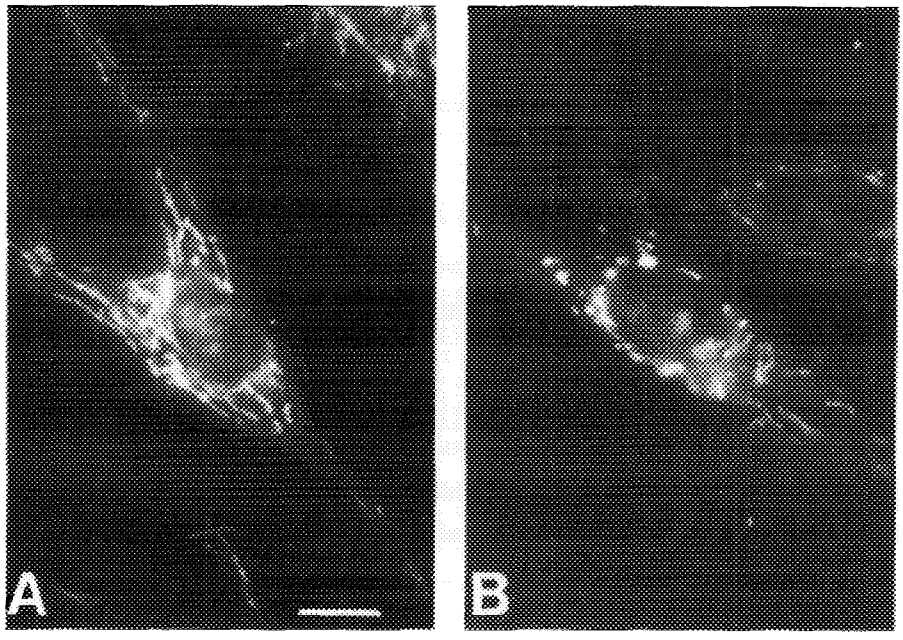


Figure 1. Visualization of mitochondria in control (A) and patient (B) fibroblasts by in vivo staining of the cells with the fluorescence dye DASPMI. Scale bar = 10 $\mu$m.

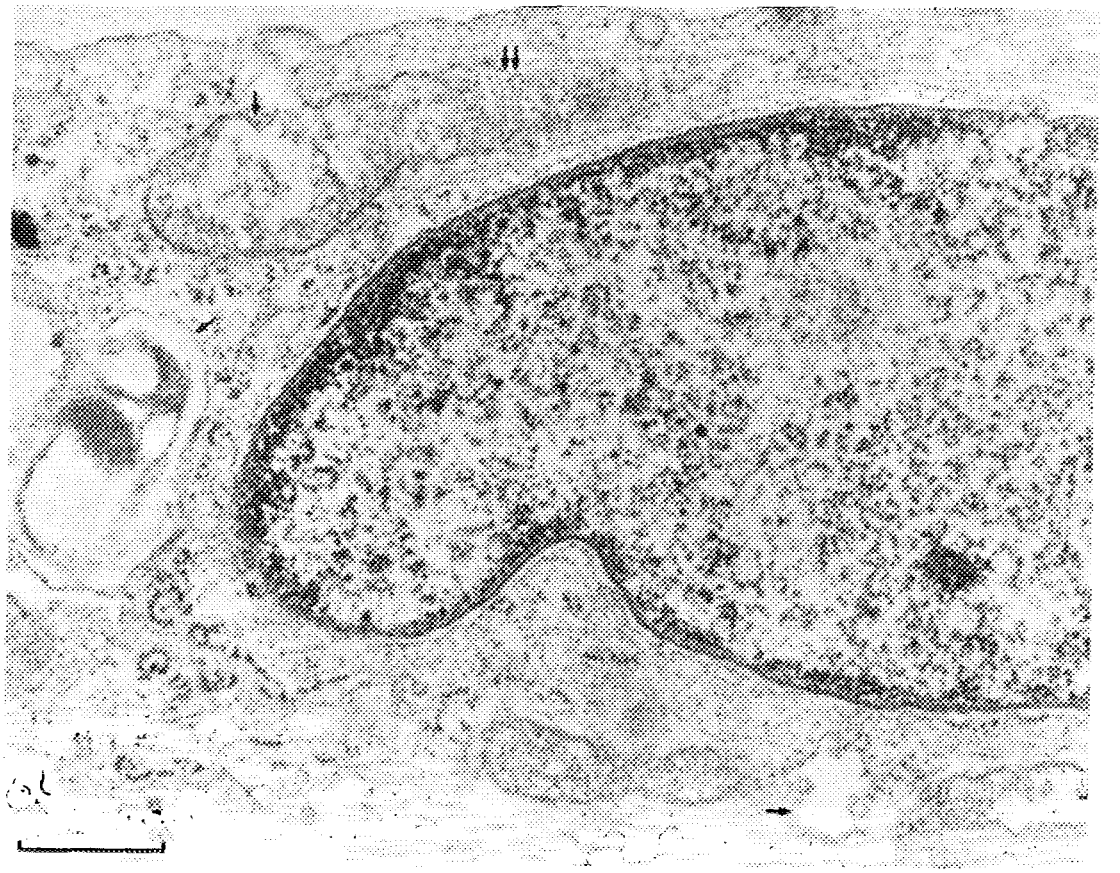

Figure 2. Electron micrograph of a patient fibroblast. The single arrows indicate sites where the inner membranes of the mitochondria are disintegrated, the double arrows a normally preserved region with intact cristae. Scale bar = 0.5 $\mu$m.

membrane to empty vesicular structures, probably due to swelling, can be seen in Figure 2. The aberrant mt structures were not preparation or fixation artefacts, since locally the mt inner membrane folding was found to be normal. Moreover, the integrity of other subcellular organelles, such as the nucleus and the endoplasmic reticulum, does affirm the good subcellular preservation.

**Levels of heat shock proteins 60 and 70.**

We have determined immunochemically the presence and amount of hsp60 and hsp70 in lysates of patient and control fibroblasts. Both heat shock proteins are components of the mt protein import machinery. Cytosolic hsp70 catalyzes the unfolding of proteins thereby enabling their transport across the membranes of mitochondria and the endoplasmic reticulum (20-22). Hsp60 is a mt matrix protein, involved in the folding and correct assembly of polypeptides into multimeric enzymes (20-23). Cytosolic hsp70 was present in normal amounts in the patient

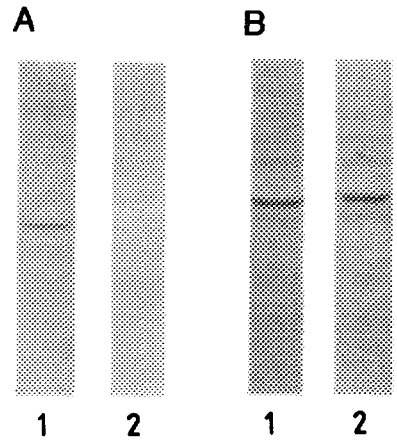


Figure 3. Immunodetection of hsp60 (A) and cytosolic hsp70 (B) on Western blots of fibroblasts from a healthy control (lanes 1) and from the patient (lanes 2). Antisera raised against yeast hsp60 and bovine hsp70 (uncoating ATPase) were used in a dilution of 1:5000.

cells (Fig. 3B). However, the amount of hsp60 determined by densitometric scanning was reduced to about one fifth of that detected in control cells (Fig. 3A). Identical results were obtained irrespective as to whether antibodies against yeast or human hsp60 were used.

## DISCUSSION

Multiple mt enzyme defects in fibroblasts are rarely mentioned in the literature. We know of only one report by Robinson et al. (24), describing a combined deficiency of succinate cytochrome c reductase, cytochrome c oxidase and pyruvate dehydrogenase. The fibroblasts from our patient showed decreased activities of 10 mt enzymes belonging to the citric acid cycle, pyruvate metabolism, oxidative phosphorylation, beta-oxidation, fatty acid transport and amino acid catabolism, respectively. Such an aspecific decrease of mt enzyme activities suggests a common defect in the maturation of these enzymes. Loss of organelle-related enzyme activities has been described earlier for peroxisomes in Zellweger Syndrome (25), a disorder of the biogenesis of peroxisomes. In some cases of Zellweger Syndrome, these pathological findings could be attributed to deficiency of PMP70, a peroxisomal membrane protein involved in transport of proteins across the membrane (26). Analogously, a defect in mt protein import or assembly could account for the defects observed in the fibroblasts of our patient. Of the proteins involved in mt import processes, hsp70 was present in normal amount,

but hp60 was found to be markedly reduced. A partial deficiency of hsp60 as the cause of this mt disorder is compatible with the following observations:

1. The defect was systemic and therefore, presumably affected all cells containing mitochondria.
2. The mt enzymes with decreased activities are multimeric and located in the mt inner membrane/matrix compartment. Also the structural defects were confined to this compartment.
3. The activity of carnitine palmitoyltransferase I was found to be normal. This enzyme is located in the outer mt membrane (27). Maturation therefore, does not require hsp60-mediated assembly.
4. As hsp60 is encoded by a nuclear gene, Mendelian inheritance of the defect had to be expected. The family history of our patient suggests an autosomal recessive mode of inheritance.

We consider a hsp60 deficiency the preferred explanation for the disease in this patient and her sister, although it can not be completely excluded that the primary defect resides in another protein of the mt protein import machinery. Defects of one of the proteins mediating mt protein import or assembly represent a new class of mt disorders, and may account for cases with lactic acidosis and multiple mt defects in fibroblasts.

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