

Deficiency of the Voltage-Dependent Anion Channel: A Novel Cause of Mitochondriopathy

MARJAN HUIZING, WIM RUITENBEEK, FRIEDRICH P. THINNES, VITO DePINTO, UDO WENDEL, FRANS J. M. TRIJBELS, LEO M. E. SMIT, HENK J. TER LAAK, AND LAMBERT P. VAN DEN HEUVEL

Departments of Pediatrics [M.H., W.R., U.W., F.J.M.T., L.P.v.d.H.] and Neurology [H.t.L.], University Hospital, Nijmegen, The Netherlands; Department of Immunochemistry, Max-Planck-Institute for Experimental Medicine, Göttingen, Germany [F.P.T.]; Institute of Biochemical and Pharmacological Sciences, University of Catania, Catania, Italy [V.D.P.]; and Department of Child Neurology, Free University Hospital, Amsterdam, The Netherlands [L.M.E.S.]

ABSTRACT

A patient with a deficient voltage-dependent anion channel (VDAC) is reported, presenting clinically with psychomotor retardation and minor dysmorphic features. Biochemical studies on muscle mitochondria showed impaired rates of pyruvate oxidation and ATP production; however, no specific deficient activity of one of the mitochondrial enzymes was involved. Western blotting experiments indicated an almost complete VDAC deficiency in skeletal muscle. The only moderately decreased VDAC content in the patient's fibroblasts might indicate that VDAC is expressed in a tissue-specific manner. The deficiency is likely caused by a mutation in the HVDAC1 gene or by

a disturbed posttranslational modification. This is the first described deficiency of a component of the outer mitochondrial membrane associated with the pyruvate oxidation pathway. Defects in this membrane should be considered as a possible cause of otherwise unexplained mitochondrial disorders. (*Pediatr Res* 39: 760-765, 1996)

Abbreviations

ANT, adenine nucleotide translocator
CCCP, carbonyl cyanide-*p*-(trifluoromethoxy)phenylhydrazone
VDAC, voltage-dependent anion channel

A dramatic increase in recognition of disorders of mitochondrial energy metabolism has been seen in the last decade (1, 2). These disorders show diverse clinical symptoms ranging from pure myopathy with lactic acidosis to severe multisystem disease with central nervous system involvement. Frequently, biochemical defects of the pyruvate dehydrogenase complex, or of one or more of the four multisubunit complexes of the respiratory chain, have been demonstrated as underlying causes (1, 3), or they are associated with mutations in the mitochondrial genome (1, 2).

Biochemical investigations in patients suspected to have a respiratory chain defect are commonly performed on skeletal muscle. They may involve measurement of substrate oxidation or oxygen consumption by intact mitochondria to obtain information about the overall flux through the various parts of the mitochondrial energy metabolism. In case of an impaired flux, the different enzyme components of the respiratory chain and of the pyruvate dehydrogenase complex are measured, possibly

followed by immunoblotting to precisely define the defect at the enzyme and protein level.

Despite progress in the diagnostic approach, we experienced that, in almost a quarter of the patients with evidently reduced substrate oxidation in muscle mitochondria, these abnormalities could not be ascribed to a known defect in one of the mitochondrial enzymes. We hypothesized that, in at least part of this category of patients, malfunctioning of one of the transport mechanisms across the mitochondrial membranes may cause biochemical and clinical disorders.

For adequate functioning of the mitochondrial energy pathway, metabolites which are directly involved in the process of oxidative phosphorylation, such as ADP, ATP, and phosphate have to be transported across mitochondrial membranes, as well as ions (K^+ , Na^+ , H^+ , Ca^{2+}) to maintain osmotic homeostasis in the mitochondrial matrix (4, 5). Hence, when studying mitochondrial disorders it seems worthwhile to investigate the proteins which are responsible for the transport of the different substances across mitochondrial membranes.

VDAC is one of these transporters in the outer mitochondrial membrane. This channel, also called mitochondrial porin, forms a pore which at low transmembrane voltage is open for anions such as phosphate, chloride, and adenine nucleotides.

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Correspondence: Marjan Huizing, Department of Pediatrics, University Hospital Nijmegen, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands.

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At a high transmembrane voltage or in the presence of a specific VDAC-modulating protein, VDAC functions as a selective channel for cations and various uncharged molecules (6, 7).

To detect disorders of the mitochondrial transport system, we screened a selected group of patients for abnormalities of VDAC in skeletal muscle. One subject with deficiency of VDAC protein was detected. The preliminary findings of this patient have previously been published (8). The clinical, biochemical, and muscle morphologic features of the patient are described in the present report.

METHODS

Case report. The male patient, the third child of healthy unrelated parents, was born at term after a normal pregnancy. Delivery of the macrosomic child (weight 5.7 kg, length 60 cm, head circumference 43.2 cm) was delayed. The patient presented with mild unspecific facial dysmorphism with prominent forehead, deep nasal bridge, mild hypertelorism, and high arched palatum. The internipple distance was increased, and the hypotonic abdominal wall showed median diastasis and herniation of the umbilical cord. There was a bilateral syndactyly of the 2nd and 3rd toe with hypoplastic nails. Initially due to intrapartum asphyxia, resuscitating measures were required. The Apgar score was 7 after 10 min. Oxygen was administered during the first 10 postnatal days. Brief neonatal seizures subsided after the start of phenobarbital treatment. In the first weeks mild hypothalamic hypothyroidism became obvious, and treatment with thyroxin was started. The psychomotor development was abnormal; the patient showed failure to thrive and had persistent muscular hypotonia with persistent head lag. There was poor visual fixation and no progressive development or milestones.

At the age of 5 mo, a magnetic resonance imaging scan of the brain showed mildly dilated and disfigured lateral ventricles (Fig. 1a). An area of cortical dysplasia, probably polymicrogyria, was seen in the region of the right Sylvian fissure (Fig. 1, a and b). The entire cortex showed too many unbranched gyri. The white matter contained hardly any myelin and had a high signal intensity throughout, indicative of a delay in myelination.

At the age of 7 mo a progressive hydrocephalus was diagnosed and treated with ventriculo-peritoneal drainage. Electroencephalography showed epileptic discharges. Visual and auditory evoked potentials showed reduction of central conduction velocities. Motor conduction velocities were normal. Electromyography revealed a myopathic pattern. The patient was strongly suspected to suffer from the Pallister-Killian syndrome, but a tetrasomy 12p was excluded both in blood cells and fibroblasts. The results of repeated routine laboratory tests in blood, cerebrospinal fluid, and urine were within normal limits, and comprehensive metabolic investigations gave normal results as well. Because an i.v. glucose loading test (2 g/kg body weight) showed a pathologic increase in blood lactate concentration (from 1.1 to 2.4 mM), a mitochondrial respiratory chain defect was considered in the differential diagnosis. Therefore a muscle biopsy of the quadri-

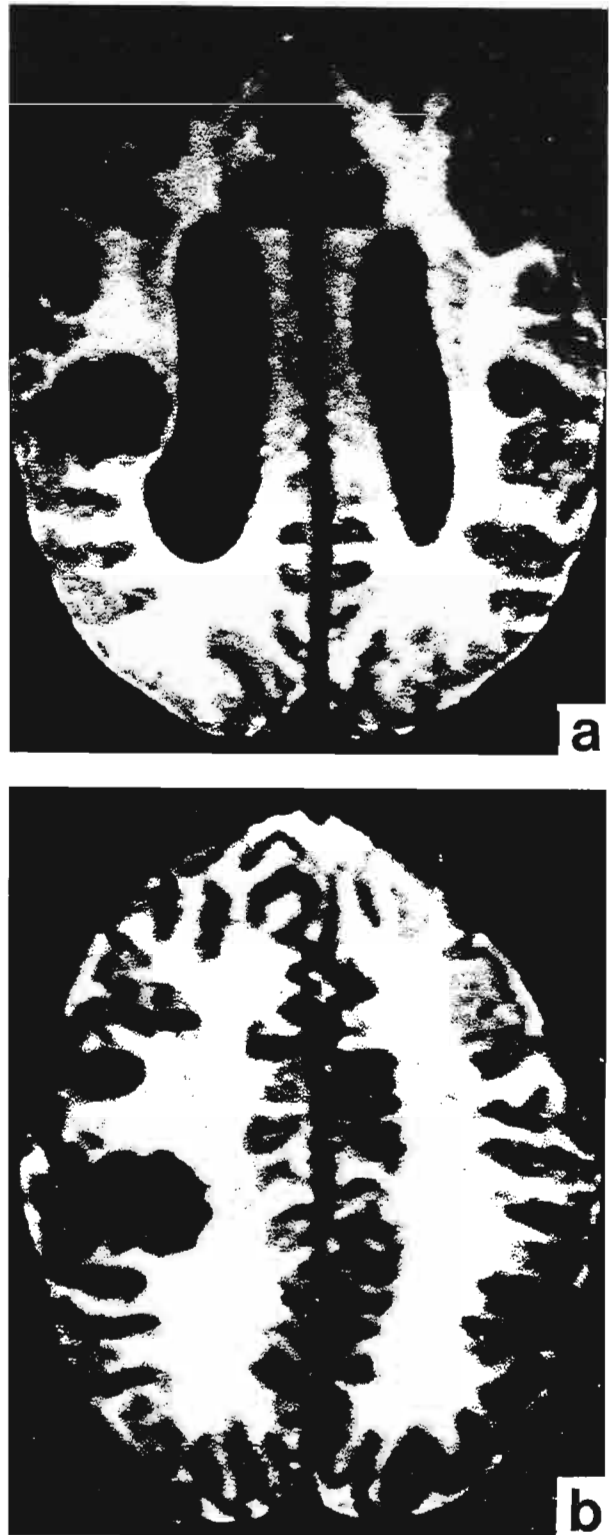


Figure 1. Proton density (a) and T_2 -weighted (b) transverse magnetic resonance images of the patient's brain, obtained at the age of 5 mo.

ceps was performed at the age of 20 mo. At the age of 4 y the main clinical symptom was psychomotor retardation.

Morphologic studies. For morphologic investigations 8- μ m sections of skeletal muscle were used for conventional histology and enzyme histochemistry. The sections were stained for succinate dehydrogenase, myosin-ATPase (pH 4.3, 4.6, and

10.3). and cytochrome oxidase according to standard procedures (9, 10). A small muscle specimen was fixed in glutaraldehyde for electron microscopic studies.

Biochemical studies. For biochemical investigations 10% (wt/vol) muscle homogenate was prepared in SETH medium (250 mM sucrose, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4, 50 U/mL heparin). The $600 \times g$ supernatant was used for biochemical determinations (11). Energy metabolism of intact muscle mitochondria was tested by measuring the rates of oxidation of labeled substrates and of ATP plus creatine phosphate production as described earlier (11). [^{14}C]Pyruvate, [$U-^{14}C$]malate, and [$1,4-^{14}C$]succinate (1 mM) were used in combination with different co-substrates and specific inhibitors. In parallel experiments mitochondrial ATP and creatine phosphate production from pyruvate as well as from succinate was measured. All incubations were performed in the presence of 2 mM ADP and 20 mM creatine. In one experiment pyruvate oxidation was determined in the presence of the uncoupling agent CCCP (2 μ M). Activities of the various enzyme complexes of the respiratory chain were determined in the $600 \times g$ supernatant as previously described (11). Samples were frozen and stored at $-80^{\circ}C$ after homogenization of fresh muscle. Activity of the pyruvate dehydrogenase complex and content of carnitine were determined in total muscle homogenate (12, 13).

Skin fibroblasts were cultured in Medium 199 with Earle's salts (ICN Biomedicals, Zoetermeer, The Netherlands) to confluency. The lactate/pyruvate ratio in fibroblasts was determined essentially as described by Wijburg *et al.* (14).

For all other studies on fibroblasts, mitochondrial enriched fractions prepared from cultured fibroblasts by homogenization and subsequent differential centrifugation were used.

Immunochemical studies. Muscle $600 \times g$ supernatants (11) and isolated mitochondria ($14,000 \times g$ pellet of the $600 \times g$ fraction) from 70 suspected patients, as well as mitochondrial enriched fractions of skin fibroblasts, were used for immunochemical studies. The samples were suspended at room temperature in sample buffer [4% (wt/vol) SDS, 5 mM aminobenzidine, 6 M urea, 0.01% (wt/vol) bromphenol blue in 6.25 mM Tris-HCl, pH 6.8]. Samples containing 100 mU of the mitochondrial reference enzyme cytochrome *c* oxidase (unless otherwise indicated), determined according to the method of Cooperstein and Lazarow (15), were applied to 10–15% polyacrylamide gels. The proteins in the samples were separated by SDS-PAGE and electroblotted to 0.2- μ m nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Both electrophoresis and electroblotting were performed on Phast System equipment (Pharmacia-LKB, Woerden, The Netherlands). The blots were incubated with various antisera against ANT and VDAC. Antigen-antibody complexes were detected using enhanced chemiluminescence (ECL: Amersham International plc, Buckinghamshire, England) with biotinylated horseradish peroxidase as detection ligand (Dako, Glostrup, Denmark).

Antibodies. Two polyclonal antisera against ANT were applied to Western blots. One was raised against ANT isolated from bovine heart (16) and one against the synthetic peptide corresponding to the C-terminal part (amino acids 288–297) of the protein (17). Three different antibodies against VDAC were

applied: one polyclonal antiserum against VDAC isolated from bovine heart (18) and two MAb raised against VDAC isolated from human B lymphocytes and cultured in two different cell lines (MAb-4 and MAb-6) (19).

RESULTS

Morphology. Examination of the muscle by light microscopy revealed a normal checkerboard pattern of fiber types with 45% type I and 55% type II fibers. The fiber diameter was smaller (8–19 μ m) compared with age-matched controls (10–30 μ m). Other stainings (*i.e.* succinate dehydrogenase, cytochrome oxidase, NADH-dehydrogenase, ATPase) gave normal patterns. Ultrastructural investigations showed normal mitochondria with clearly visible mitochondrial outer membranes (Fig. 2).

Biochemistry. In muscle all measured substrate oxidation rates were decreased (Table 1). Uncoupling by CCCP did not stimulate pyruvate oxidation. The generation of high energetic compounds was decreased to the same extent as the oxidation rates. The activities of the respiratory chain enzymes were related to the mitochondrial reference enzyme citrate synthase because the mitochondrial content of human skeletal muscle can vary to a considerable extent, especially in pathologic tissue. One should distinguish between lowered mitochondrial content and a specific enzyme deficiency. No specific deficiency of a respiratory chain enzyme was detected. The activities of the respiratory chain enzymes were all normal (Table 1). The carnitine content in muscle was slightly decreased: 2.1 μ mol/g of muscle [control (n) = 2.7–4.7].

In patient's fibroblasts the lactate/pyruvate ratio after glucose conversion appeared to be normal: 12 ($n < 25$), which means there is no indication for a disturbance in oxidative phosphorylation in fibroblasts.

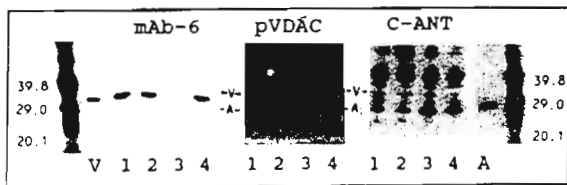
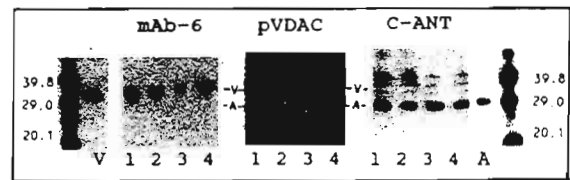
Immunochemistry. A clearly decreased amount of the VDAC protein (35 kD) in the patient's muscle, using both MAb against human VDAC as well as the polyclonal anti-bovine heart VDAC antiserum, is seen in Figure 3. The ANT



Figure 2. Electron micrograph of patient's quadriceps muscle showing clearly the intact mitochondrial outer membrane. Bar, 0.1 μ m.

Table 1. Biochemical activities of fresh quadriceps muscle from patients and controls in $\text{nmoles} \cdot \text{hr}^{-1} \cdot \text{mU}^{-1}$ citrate synthase ($n = \text{number of controls}$)

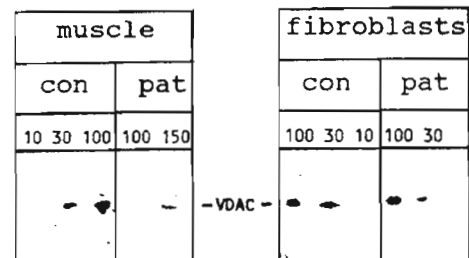
Activity	Patient	Control range	<i>n</i>
Oxidation rates for:			
[1- ¹⁴ C]Pyruvate + malate	1.15	3.61–7.48	14
[1- ¹⁴ C]Pyruvate + carnitine	1.74	2.84–8.24	14
[1- ¹⁴ C]Pyruvate + malate + CCCC	1.34	3.36–7.74	14
[U- ¹⁴ C]Malate + pyruvate + malonate	1.20	4.68–9.62	14
[U- ¹⁴ C]Malate + acetylcarnitine + malonate	1.31	3.43–7.30	14
[U- ¹⁴ C]Malate + acetylcarnitine + arsenite	0.83	2.05–3.85	14
[1,4- ¹⁴ C]Succinate + acetylcarnitine	0.86	2.54–6.39	14
ATP + creatine phosphate production rates from:			
Pyruvate + malate (arsenite sensitive)	9.9	42.1–81.2	14
Succinate + acetylcarnitine + rotenone (antimycine A sensitive)	5.9	7.2–18.4	14
Activity of:			
NADH:Q ₁ oxidoreductase	0.19	0.044–0.265	25
Succinate: cytochrome <i>c</i> oxidoreductase	0.28	0.30–0.97	25
Cytochrome <i>c</i> oxidase	1.04	0.81–3.12	25
NADH:O ₂ oxidoreductase	0.30	0.28–0.84	25
Pyruvate dehydrogenase complex	0.17	0.028–0.089	25
Citrate synthase*	28.6	48–162	25

* In $\text{mU} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein.**Figure 3.** Immunoblots of patient and control muscle incubated with antibodies against VDAC and ANT. The $600 \times \text{g}$ muscle supernatants, containing 100 mU of cytochrome *c* oxidase, were loaded in each lane. Lanes 3, patient's muscle; lanes 1, 2, and 4, three different control muscles; lane V, isolated VDAC (35 kD); lane A, isolated ANT (29 kD). Outer lanes, biotinylated molecular mass marker (39.8, 29.0, and 20.1 kD). The immunostaining was performed with three different antibodies: monoclonal anti-VDAC (*mAb-6*), polyclonal anti VDAC (*pVDAC*), and polyclonal anti C-terminal ANT (*C-ANT*).**Figure 4.** Immunoblot of mitochondrial enriched fractions of cultured skin fibroblasts from patient (lanes 3) and three different controls (lanes 1, 2, and 4), all containing 100 mU of cytochrome *c* oxidase. Immunostaining was performed with antibodies against VDAC (*mAb-6*, *pVDAC*) and ANT (*C-ANT*). For additional details, see legend to Figure 2.

content (29 kD) appears to be only slightly reduced. The polyclonal anti-VDAC antiserum also reacts with other muscle proteins. In this polyclonal anti-VDAC panel the amount of all proteins in the patient appeared to be comparable with those in controls except for the VDAC protein. The polyclonal antiserum against total ANT gave similar results (not shown) as the antiserum directed against the C terminus of ANT. The two MAb against VDAC isolated from human B lymphocytes (MAb-4 and MAb-6) reacted comparably in all applications. In the Figures 3, 4, and 5, only MAb-6 results are shown.

Figure 4 shows the results of the immunochemical examination of skin fibroblasts. Compared with muscle tissue the patient's fibroblasts show a less decreased VDAC content. The amount of ANT in fibroblasts is normal compared with controls.

The relative VDAC content in the patient's muscle tissue and fibroblasts is presented in Figure 5. The specific amount of VDAC is about 10-fold decreased in the patient's muscle, whereas in fibroblasts the VDAC amount appears to be only slightly decreased.

**Figure 5.** Investigation of the amount of VDAC in the patient's muscle (left) and fibroblasts (right) by using varying activities of the mitochondrial reference enzyme cytochrome *c* oxidase (10, 30, 100, and 150 mU). The activity of cytochrome *c* oxidase in milliunits and source of the samples (*con.* control; *pat.* patient) are indicated at the top. The blots were incubated with MAb-6 antiserum.

DISCUSSION

At present, knowledge about the function of mitochondrial membrane carriers in human physiology and pathophysiology is limited. Only a few subjects with a defective carrier have been described: one with ANT deficiency (20), one suspected to have a defective protein import into mitochondria (21), and one likely with a defective malate-aspartate shuttle (22). Lack of the carnitine carrier has been found more frequently (23). To our knowledge no systematic study has been performed to identify deficiencies of other mitochondrial membrane carriers.

By screening a selected group of subjects, we found one with deficient VDAC protein in skeletal muscle. In this subject, investigation of the muscle mitochondria showed impaired substrate oxidation and reduced production of high energetic compounds (Table 1). These data point to a functional defect in one of the many components involved in the mitochondrial energy metabolism, *i.e.* respiratory chain enzymes or postrespiratory chain energy-converting steps, such as ATPase, mitochondrial creatine kinase, or ANT. An abnormal mitochondrial matrix composition, due to altered ion homeostasis or diminished NAD^+ or coenzyme A concentration, could also have resulted in a reduced oxidative phosphorylation capacity.

A defect in the respiratory chain could be excluded by demonstrating normal enzyme activities. A defect in postrespiratory chain energy-converting processes was less presumable because stimulation of pyruvate oxidation by use of the uncoupler CCCP, which is expected in such a case (20, 24), failed to appear.

In contrast, abnormal ion composition and/or altered osmolarity within the mitochondrial matrix could be a logic explanation for an impaired mitochondrial energy metabolism in our patient. In this respect it is well known that a lower pH in the mitochondrial matrix resulting from any disturbance of proton transport across mitochondrial membranes inhibits mitochondrial ATPase and thus ATP production (25), as well as various components of the respiratory chain and mitochondrial transport systems (26). Likewise, maintenance of the volume of the mitochondrial matrix within a narrow range is crucial for proper energy metabolism. Any change of the permeability of the inner or outer mitochondrial membrane may influence the respiratory rate (4, 5, 7).

One of the proteins involved in anion and cation transport across the outer mitochondrial membrane is VDAC. VDAC is an abundant channel-forming protein; the pore size is regulated by the membrane potential (6, 7, 27, 28). The shortage of VDAC in our patient's muscle tissue might have caused an abnormal ion composition of the mitochondrial matrix, thereby deteriorating the process of oxidative phosphorylation. Because VDAC is also responsible for the transport of ATP and ADP across the outer mitochondrial membrane (6, 27), one would expect in our patient a reduced transport of ADP and inorganic phosphate inward, as well as ATP out of the mitochondria, thereby hampering the energy metabolism. From the fact that the uncoupler CCCP did not stimulate substrate oxidation in muscle tissue, it could be hypothesized that biochemical abnormalities in VDAC-deficient mitochondria have been the consequence of malfunctioning of ion transport rather than the consequence of a blockade of ADP and ATP transport.

The important role of VDAC in energetic and metabolic function of mitochondria is illustrated with VDAC-deficient mutants, which have been generated only in yeast mitochondria. These mutants showed a disturbed respiration, whereas the respiration in the uncoupled state was normal. The mutants showed an increased requirement for ADP and Mg^{2+} ions (29–31). To comply with the requirements, the size of VDAC pores may be regulated, either by a specific modulator protein (7, 32), by NADH concentration, or by the colloidal osmotic pressure, shifting the membrane potential required for opening

and closure of the pores (5, 33–35). Also the fact that VDAC interacts with kinases, ANT, and benzodiazepine receptors in important mitochondrial structures, such as special contact sites and megachannels, might underline its regulatory role in mitochondrial energy metabolism (5, 27, 35, 36).

In our patient VDAC deficiency was expressed in a tissue-specific manner. VDAC protein was severely decreased in skeletal muscle, but only moderately decreased in cultured fibroblasts. With respect to human VDAC, five partly established cDNA sequences, encoded by four genes, have been reported (37–39). The occurrence of isoforms might be an explanation for the tissue-specific VDAC disturbances in our patient. However, the monoclonal anti-VDAC antibody which was used in our experiments was shown to recognize only the HVDAC1 isoform and more specifically the acetylated N-terminal region (40). So, the most likely explanation is that the HVDAC1 protein in muscle tissue of our patient is almost completely absent, mutated, or nonacetylated. The gene of HVDAC1 is located on chromosome X in the interval Xq13-q21 (38). High resolution analysis of the patient's X chromosome did not demonstrate abnormalities. Studies are in progress to detect the defect at molecular level.

In our patient, a damage of the mitochondrial outer membrane in which VDAC is incorporated could also have induced a lack of the VDAC protein. However, ultrastructural investigations clearly showed the presence of a normal mitochondrial outer membrane. Unfortunately, not enough muscle was available to estimate the presence of other components of the mitochondrial outer membrane. In our opinion the VDAC deficiency is not caused by the absence of (large parts of) the outer membrane, because oxidative phosphorylation remains usually unimpaired in mitochondria devoid of the outer membrane (5, 6, 31). Removal of the mitochondrial outer membrane in VDAC-deficient mutants even induces normalization of energy metabolism (31).

VDAC has also been reported to be a constituent of the plasma membrane of mammalian brain cells (41, 42), human T and B lymphocytes (19, 43, 44), muscle cells (19), and an epithelial carcinoma cell line (45). It seems to occur also in the sarcoplasmic reticulum (46). It remains obscure whether the multitopologic localization of VDAC and its deficiency is of importance for the clinical findings in our patient. It is unknown whether VDAC is also deficient in cerebral tissues and whether the abnormalities in magnetic resonance imaging findings and psychomotor development are related to such a defect.

In conclusion, this report on a patient with a deficient VDAC protein together with a recently reported ANT defect (20) suggests that an immunochemical approach provides a useful extension of the diagnostic program for detecting mitochondrial disorders.

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