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Brief Communication

Fabry disease: reduced activities of respiratory chain enzymes with decreased levels of energy-rich phosphates in fibroblasts

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Abstract

Fabry disease (FD, MIM 301500) caused by a deficient activity of α-galactosidase A is characterized by intralysosomal storage of glycosphingolipids. Main clinical features are paresthesia, hypohidrosis, angiokeratoma, renal insufficiency, and cardiovascular or cerebral complications. The exact pathogenesis is unclear. Beside mechanical storage biochemical factors might play a role. As FD is a multisystemic disorder and mitochondrial dysfunction has been described in patients with neuronal ceroidlipofuscinosis (another lysosomal storage disease) we examined mitochondrial function in fibroblasts from patients with FD.

Results. Activities of respiratory chain enzymes I, IV, and V were significantly ($p < 0.01$) lower in FD-cells. Mitochondrial recovery was unchanged as judged by the activity of the mitochondrial marker enzyme citratesynthase, cellular protein content was not significantly different. CP, ADP, and AMP concentrations were significantly ($p < 0.01$) lower in FD-cells. ATP was slightly, but not significantly reduced ($p = 0.045$).

Conclusion. Organ dysfunction in FD may not only be explained by mechanical storage of glycosphingolipids. As in NCL, lysosomal storage material may lead to mitochondrial dysfunction with a reduction of respiratory chain enzyme activities and a subsequent drop in cellular levels of energy-rich phosphates.
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Introduction

Fabry disease (FD, MIM 301500)—also known as Anderson disease—is a pan-ethnic, X-linked lysosomal storage disorder (LSD) caused by a deficiency of the lysosomal enzyme α-galactosidase A (α-Gal A). A defect of α-Gal A (encoded on an X-linked gene in the region Xq22.1) leads to disturbance in the biodegradation of sphingoglycolipids, resulting in progressive deposition of terminal galactosyl-containing neutral glycosphingo-lipids in lysosomes [1].

The main clinical symptoms in hemizygous patients with FD are painful episodic crises, acroparesthesias, angiokeratomas, gastrointestinal symptoms, corneal opacities, microvascular disease of the kidneys, heart, and brain which often lead to premature death. Hetero-zygous females often have a milder course of the disease with similar symptoms. The pathogenesis in FD is still unknown. As mitochondrial energy supply has a central role in almost all cells disturbed mitochondrial function may play a role in this multisystemic disorder. Diastolic dysfunction of the heart is a frequent finding in patients with the cardiac variant of FD [2]. Diastolic relaxation requires cellular energy, therefore compromised mito-chondrial function may be responsible for diastolic dys-function in FD. In another lysosomal storage disease, the neuronal ceroidlipofuscinosis (NCL), impaired mito-chondrial function has been demonstrated [3,4]. In previ-ous studies, our group has demonstrated abnormal activity of the mitochondrial ATPsynthase resulting in decreased intracellular levels of energy-rich phosphates in NCL [5,6]. Thus, the concept that only mechanical factors play a role in the pathogenesis of FD seems sim-plistic. These findings prompted us to examine mito-chondrial function in FD. In the present study we measured the activities of respiratory chain enzymes and

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the cellular contents of high-energy phosphate compounds (creatinephosphate CP, ATP, and ADP) and AMP in fibroblasts from patients with FD. Deposition of storage material has been demonstrated in human fibroblasts [7–9].

Materials and methods

Cultured skin fibroblasts from four patients with Fabry disease and 10 healthy age-matched controls were examined. Cells were from early passage numbers. Fibroblasts from patients with Fabry disease (GM002775/GM02769/GM00882/GM0107) were purchased from NIGMS Human Genetic Cell Repository, Coriell Institute for Medical Research, Camden, USA. Fibroblasts were plated on Petri dishes (diameter 30 mm) and grown to confluence.

For spectrophotometric and luminometric experiments cells were washed twice with a Hepes buffer containing (mM) NaCl 110, KCl 2.6, KH_2PO_4 1.2, $CaCl_2$ 1, $MgSO_4 \cdot 7H_2O$ 1.2, Hepes 25, pH 7.4, and then incubated with 10 mM glucose for 15 min at room temperature.

Respiratory chain enzyme activities were assayed as described before [10,11]. Cells were broken by sonication (2×10 s) using a BANDELIN probe sonicator set at 20 W of power with single pulses of 0.3 s duration. Time between pulses was 0.7 s.

The activities of mitochondrial complexes I + III were measured spectro-photometrically at a wavelength of 340 nm and a temperature of 37 °C with rotenone serving as specific inhibitor of complex I [12]. The activities of complexes II + III were determined at 550 nm and 37 °C. The reaction was inhibited by antimycine A [13]. The enzyme activity of complex IV was measured at a wavelength of 550 nm and 37 °C [14]. The activity of complex V (oligomycine-sensitive ATPase) was assayed spectrophotometrically at 340 nm and 37 °C [15,16].

For luminometric experiments an aliquot of the cell homogenate was placed in an equal volume of ice-cold DMSO (dimethylsulfoxide) as described by Ouhabi [17,18]. High energy phosphate compounds were measured in triplicate at room temperature via bioluminescence in a Bioorbit-luminometer using a luciferin/luciferase assay. ATP and creatinephosphate were assayed according to Ronner et al. [19]. ADP using pyruvate kinase as a coupling enzyme was measured as described elsewhere [20].

Protein was assayed according to Bensadoun and Weinstein [21].

Materials

Tissue culture materials were from Biochrom (Berlin, Germany), FALCON 3001 tissue culture dishes came from Becton–Dickinson (Heidelberg, Germany). All chemicals used were from Sigma–Aldrich (Taufkirchen, Germany). Enzymes were from Roche (Mannheim, Germany). BIOORBIT test kits for luminometric measurements were obtained from Labsystems (Turku, Finland).

Statistical analysis

Data from controls and FD cells were compared using Student's t-test for unpaired data.

Results

Morphology of fibroblasts from patients with FD was unchanged compared to control cells as judged by light microscopy. Cellular content of protein was not significantly different between controls and FD cells.

Measurements of respiratory chain enzyme activities were carried out in triplicate, variation between determinations from the sonicate of one Petri dish was typically 5%, variation between different Petri dishes from the same cell line was <10%. The activities of respiratory chain enzymes I–V in control fibroblasts versus fibroblasts of patients with FD are shown in Table 1. Activities of respiratory chain enzymes I + III, IV, and V were significantly ($p < 0.01$) lower in FD cells. Complex II+III—activity was not significantly reduced ($p = 0.05$). The activity of the mitochondrial marker enzyme citratesynthase was normal in both groups of cells. When the activities of respiratory chain enzymes were normalised to citratesynthase activities of complexes I+III, IV, and V were still significantly reduced in FD cells as compared to controls (Table 2).

Assays of high-energy phosphates were carried out in triplicate, variations between measurements from the same sonicate as well as from the same cell line were similar to determinations of respiratory chain enzyme activities. The contents of ATP, ADP, AMP, and CP in the two different cell preparations are summarized in Table 3. Compared

Table 1
Activities of respiratory chain enzymes (nmol/min per mg protein) mean ± SD

	Complex I + III	Complex II + III	Complex IV	Complex V	Citrate-synthase
Controls ($n = 10$)	104 ± 13	7 ± 1	56 ± 7	140 ± 23	11 ± 6
Fabry disease ($n = 4$)	25[*] ± 11	4 ± 0.1	14[*] ± 6	48[*] ± 21	10 ± 2

[*] $p < 0.01$.

Table 2
Activities of respiratory chain enzymes normalised to citratesynthase activities, mean ± SD

	Complex I + III/CS	Complex II + III/CS	Complex IV/CS	Complex V/CS
Controls ($n = 10$)	10.0 ± 1.8	0.5 ± 0.2	4.7 ± 1.2	12.0 ± 3.2
Fabry disease ($n = 4$)	2.6[*] ± 1.3	0.4 ± 0.1	2.1[*] ± 0.9	4.6[*] ± 2.1

[*] $p < 0.01$.

Table 3
Contents of ATP, ADP, AMP, and CP (nmol/mg protein) in control fibroblasts and cells from patients with Fabry disease (FD)

	ADP		AMP		ATP		CP	
	Controls $n = 12$	FD $n = 4$	Controls $n = 12$	FD $n = 4$	Controls $n = 12$	FD $n = 4$	Controls $n = 12$	FD $n = 4$
Mean ± SD	4.4 ± 2.4	3.1[*] ± 1.1	2.2 ± 1.5	1.4[*] ± 0.58	16.2 ± 6.1	15.6 ± 5.0	10.8 ± 5.4	8.7[*] ± 2.8

[*] $p < 0.01$.

with control cells the concentration of ATP was slightly but not significantly reduced in FD cells ($p = 0.045$). Concentrations of ADP and AMP as well as CP were significantly reduced ($p < 0.01$) in FD fibroblasts.

Discussion

In the present study we found reduced activities of respiratory chain complexes in fibroblasts from patients with FD, whereas the activity of the mitochondrial marker enzyme was not altered compared to the control group showing normal mitochondrial recovery. Cellular content of protein was similar in FD- and control cells. Furthermore, we found a decrease in energy-rich phosphates with ATP being mildly and ADP and CP being profoundly reduced in FD fibroblasts. This indicates that the reduced activities of respiratory chain complexes are of relevance for energy production in the intact cell. ATP-depletion was probably partially prevented by an ATP-supply via CP which serves as an 'energy buffer' within the cell using the creatinekinase reaction. The concept that mechanical deposition of storage materials in FD is directly responsible for clinical symptoms is probably simplistic. A compromised mitochondrial energy supply via oxidative phosphorylation may play a role in the pathogenesis of organ dysfunction in patients with FD. Deficient cellular energy supply may be directly responsible for impaired function of most organs affected in FD, in the CNS, however, 'secondary excitotoxicity' ('slow onset' neurodegeneration) via opening of NMDA-receptor associated ion channels [22] may be involved.

Recently, we have found compromised mitochondrial function in another lysosomal storage disease. It could be demonstrated that the ATPsynthase activity was reduced in fibroblasts from children with late-infantile and juvenile NCL [23]. Furthermore, we found anomalies in the up-regulation of the mitochondrial ATPsynthase in

fibroblasts from children with the infantile form of NCL as well as in an ovine model [5]. These findings were of relevance in the intact cell as levels of high-energy phosphate compounds were altered in NCL-fibroblasts [6].

The reason for the reduction of respiratory chain enzyme activities in FD, however, remains unclear. An allosteric factor leading to decreased activities of respiratory chain enzymes can be excluded as inhibition was stable to dilution into large volumes during and after sonication of cells. We rather suspect that hydrophobic storage material might change biophysical properties of the inner mitochondrial membrane. ATPsynthase has been shown to be actively regulated in cultured human fibroblasts [10,24]. A regulatory element is the naturally occurring inhibitor protein IF1 [25] which binds to the ATPsynthase when the potential across the inner mitochondrial membrane is decreased [26]. The membrane potential may be decreased by increasing the dielectric constant and/or decreasing the thickness of the inner mitochondrial membrane or by raising the permeability of the membrane. All these factors would promote binding of IF1 to the ATPsynthase molecule which could explain decreased activity of complex V in FD fibroblasts. ATPsynthase-regulation plays an important role under physiological and pathophysiological conditions [24]. Compromised activity of the ATPsynthase may lead to secondary inhibition of other respiratory chain complexes either by a lack of energy or by accumulation of reactive oxygen species (ROS). On the other hand, respiratory chain complexes are embedded in the lipid milieu of the inner mitochondrial membrane. Therefore, abnormal lipophilic storage material in the inner mitochondrial membrane may directly interact with respiratory chain complexes or alter structural proteins in the inner mitochondrial membrane. This would result in an impaired electron flux across the respiratory chain resulting in a decreased membrane potential across the inner mitochondrial membrane. Subsequently, IF1 would bind to the ATPsynthase

leading to secondary inhibition of complex V [16]. Cardiolipin is the most abundant substance in the inner mitochondrial membrane and may be qualitatively or quantitatively altered by lipophilic storage material in FD. It is not clear why the complex II + III activity is not decreased to the same extent as the activities of complexes I, IV, and V.

In a previous study it has been shown that isolated mitochondria can be uncoupled and oxidative phosphorylation can be inhibited after incubation with lysosomal storage material in vitro [27]. These findings would support our thesis of mitochondrial alteration in FD.

Abnormal metabolites in blood and urine indicating mitochondrial dysfunction (e.g., hyperlactatemia, increased lactate/pyruvate ratio, increased ketone bodies and ketone body ratio, and dicarboxylic aciduria) have not been reported in FD. This may be due to dilution if only 1 or 2 organs are involved. Alternatively, it may be reasoned that storage material is deposited in blood vessels leading to a decreased washout of abnormal metabolites from affected organs.

Impaired mitochondrial energy supply is not an unspecific finding in storage diseases as we have previously shown that respiratory chain enzymes are not altered in fibroblasts from patients with sialidosis and mucopolysaccharidosis VI [23], high-energy phosphate levels were normal in fibroblasts from children with Gaucher disease I and mucopolysaccharidosis VI [6]. Only selected storage materials may interfere with mitochondrial oxidative phosphorylation.

In conclusion we demonstrate impaired mitochondrial energy supply in skin fibroblasts from patients with FD. Compromised mitochondrial function may play a role in the pathogenesis of FD.

Acknowledgments

References

[1] R.J. Desnick, Y.A. Ioannou, C.M. Eng, α-Galactosidase A deficiency: Fabry disease, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, B. Childs, K.E. Kinzler, B. Vogelstein (Eds.), The Metabolic and Molecular Bases of Inherited Disease, eighth ed., McGraw Hill, New York, 2001, pp. 3733–3774.

[2] C. Kampmann, F. Baehner, M. Ries, M. Beck, Cardiac involvement in Anderson Fabry disease, J. Am. Soc. Nephrol. 13 (suppl. 2) (2002) 147–149.

[3] A.N. Siakotos, P.S. Blair, J.D. Savill, M.L. Katz, Altered mitochondrial function in canine ceroid-lipofuscinosis, Neurochem. Res. 23 (1998) 983–989.

[4] D.N. Palmer, I.M. Fearnley, J.E. Walker, N.E. Hall, B.D. Lake, L.S. Wolfe, M. Haltia, R.D. Martinus, R.D. Jolly, Mitochondrial ATP synthase subunit c storage in the ceroid-lipofuscinoses (Batten disease), Am. J. Med. Genet. 42 (1992) 561–567.

[5] A.M. Das, R.D. Jolly, A. Kohlschütter, Anomalies of ATP-synthase regulation in four different types of neuronal ceroid lipofuscinosis, Mol. Genet. Metab. 66 (1999) 349–355.

[6] A.M. Das, R. Von Harlem, M. Feist, T. Lücke, A. Kohlschütter, Altered levels of high-energy phosphate compounds in fibroblasts from different forms of neuronal ceroid lipofuscinoses: further evidence for mitochondrial involvement, Eur. J. Paediatr. Neurol. 5 (2001) 143–146.

[7] K. Itoh, M. Kotani, T. Tai, H. Suzuki, T. Utsunomiya, H. Inoue, H. Yamada, H. Sakuraba, Y. Suzuki, Immunofluorescence imaging diagnosis of Fabry heterozygotes using confocal laser scanning microscopy, Clin. Genet. 44 (1993) 302–306.

[8] A. Wandall, L. Hasholt, S.A. Sorensen, Electron microscopic observations on cultured fibroblasts from Fabry heterozygotes and hemizygotes, Ultrastruct. Pathol. 3 (1982) 51–58.

[9] E. Kamensky, M. Philippart, P. Cancilla, S.P. Frommes, Cultured skin fibroblasts in storage disorders. An analysis of ultrastructural features, Am. J. Pathol. 73 (1973) 59–80.

[10] A.M. Das, D.J. Byrd, J. Brodehl, Regulation of the mitochondrial ATP-synthase in human fibroblasts, Clin. Chim. Acta 231 (1994) 61–68.

[11] A.M. Das, Regulation of mitochondrial ATP-synthase activity in human myocardium, Clin. Sci. 94 (1998) 499–504.

[12] J.C. Fischer, W. Ruitenbeek, J.M.F. Trijbels, J.H. Veerkamp, A.M. Stadhouders, R.C. Sengers, A.J. Janssen, Estimation of NADH oxidation in human skeletal muscle, Clin. Chim. Acta 155 (1986) 263–274.

[13] D.A. Stumpf, J.K. Parks, Human mitochondrial electron transport chain: assay of succinate: cytochrome c reductase in leucocytes, platelets and cultured fibroblasts, Biochem. Med. 25 (1981) 234–238.

[14] D.C. Wharton, A. Tzagoloff, Cytochrome oxidase from beef heart mitochondria, Methods Enzymol. 10 (1967) 245–250.

[15] J. Rosing, D.A. Harris, A. Kemp, E.C. Slater, Nucleotide-binding properties of native and cold-treated mitochondria, Biochim. Biophys. Acta 376 (1975) 13–26.

[16] A.M. Das, D.A. Harris;, Regulation of mitochondrial ATP-synthase in intact rat cardiomyocytes, Biochem. J. 266 (1990) 355–361.

[17] R. Ouhabi, M. Boue-Grabot, J. Mazat, ATP synthesis in permeabilized cells: assessment of the ATP/O ratios in situ, Mod. Trends Bio Thermo Kinet. 3 (1994) 141–144.

[18] A.M. Das, T. Lücke, K. Ullrich, Glutaric aciduria I: creatine supplementation restores creatinephosphate levels in mixed cortex cells from rat incubated with 3-hydroxyglutarate, Mol. Genet. Metab. 78 (2003) 108–111.

[19] P. Ronner, E. Friel, K. Czerniawski, S. Frankle, Luminometric assay of ATP, phosphocreatine and creatine for estimation of free ADP and free AMP, Anal. Biochem. 275 (1999) 208–216.

[20] D.A. Harris, Spectrophotometric assays, in: D.A. Harris, C.L. Bashford (Eds.), Spectrophotometry and Spectrofluorimetry, IRL Press, Oxford, 1988, pp. 74–77.

[21] A. Bensadoun, D. Weinstein, Assay of proteins in the presence of interfering material, Anal. Biochem. 70 (1976) 241–250.

[22] C. Ikonomidou, L. Turski, Excitotoxicity and neurodegenerative diseases, Curr. Opin. Neurol. 8 (1995) 487–497.

[23] A.M. Das, A. Kohlschütter, Decreased activity of the mitochondrial ATP-synthase in fibroblasts from children with late infantile neuronal ceroid lipofuscinosis, J. Inherit. Metab. Dis. 19 (1996) 130–132.

[24] A.M. Das, Regulation of the mitochondrial ATP-synthase in health and disease, Mol. Genet. Metab. 79 (2003) 71–82.

[25] N.M. Cintron, P.L. Pedersen, A protein inhibitor of the mitochondrial adenosine triphosphatase complex of rat liver. Purification and characterization, J. Biol. Chem. 254 (1979) 3439–3443.

[26] G. Lippe, C.M. Sorgato, D.A. Harris, The binding of the inhibitor protein is governed independently by ATP and membrane potential in ox-heart submitochondrial particles, Biochem. Biophys. Acta 933 (1988) 12–21.

[27] P.M. Strasberg, J.W. Callahan, Lysosphingolipids and mitochondrial function. II. Deleterious effects of sphingosylphosphorylcholine, Biochem. Cell Biol. 66 (1988) 1322–1332.