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PhD thesis summary

Study of Mitochondrial Ultrastructure and Functions in Selected Mitochondrial and Lysosomal Storage Disorders

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Abbreviations:

ATP	Adenosine triphosphate
CI - CIV	Complex I - IV of respiroatory chain
COX	Cytochrome c oxidase
FD	Fabry disease
GB3	Globotriceramidy
GD	Gaucher disease
HSF	Human Skin Fibroblasts
LSDs	Lysosomal storage disease
MELAS	Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes
MERRF	Myoclonic Epilepsy associated with Ragged-Red Fibers
MLD	Metachromatic leucodystrophy
mtDNA	Mitochondrial DNA
NARP	Neuropathy, Ataxia and Retinitis Pigmentóza
nDNA	Nucleic DNA
NPA	Niemann Pick A disease
TMPD	Tetramethyl phenyl diamine
TRIS	Tris(hydroxymethyl)aminomethan
tRNA	Transfer ribonucleic acid

SUMMARY

This thesis has been worked out in The laboratory for study of mitochondrial disorders (Departement of Pediatrics, 1st Faculty of Medcine, Chales university in Prague) and in cooperation with The Institute of Inherited Metabolic Disorders.

Mitochondrial disorders represent a heterogeneous group of diseases with the onset at any age from neonatal period till adulthood, mostly presented with very severe clinical courses of disease. The mammalian organism is fully dependent on mitochondrial oxidative phosphorylation system as on the major energy producer of the cell. Therefore the mitochondrial disorders affect mainly high energy demanded tissues such as brain, heart or muscle. Simillar phenotype is observed in many lysosomal storage disorders.

Despite of expanding knowledge of molecular basis of mitochondrial and lysosomal disorders, it may be still difficult to explain the exact pathogenesis of disease as well as the prognosis for patients and their families.

Mitochondrial functions affect more than just energy production; they contribute in initiation of apoptosis, in cellular calcium homeostasis, and in production of reactive oxygene species. Disturbed mitochondria become a goal of autophagy mediated by the lysosomal compartement.

The results of our study enable:

1. better understanding of the tissue specific impact of selected mtDNA mutations on the mitochondrial function in autoptic tissues

2. better understanding of the relations between mitochondrial ultrastructure and functions in selected mitochondrial and lysosomal storage disorders on level of cultured fibroblasts.

SOUHRN

Disertační práce byla vypracována v Laboratoři pro studium mitochondriálních poruch, Kliniky dětského a dorostového lékařství, 1. LF UK v Praze a ve spolupráci s Ústavem dědičných metabolických poruch, 1. LF UK v Praze.

Práce vychází z dlouhodobého zaměření pracoviště na studium mitochondriálních onemocnění a spojuje problematiku primárně mitochondriálních onemocnění a sekundárních mitochondriálních poruch u vybraných lysosomálních střádacích onemocnění.

Mitochondriální onemocnění představují skupinu závažných onemocnění s nástupem od novorozeneckého věku až do dospělosti a u většiny pacientů mají velmi vážný průběh. Mitochondrie jsou hlavním producentem energie v savčí buňce a poruchy systému OXPHOS mají zásadní dopady především na tkáně s vysokou energetickou náročností. Obdobný fenotyp je společný i pro řadu lysosomálních střádacích onemocnění.

I při známé molekulární podstatě mitochondriálních i lysosomálních onemocnění je stále obtížné odhadnout klinický průběh onemocnění i prognózu.

Funkce mitochondrií se neomezuje pouze na produkci energie, ale hraje i významnou úlohu ve spouštění apoptózy, v produkci volných radikálů, je významným místem vápníkového metabolismu a poškozené mitochondrie se stávají cílem autofagie kontrolované lysosomy.

Naše výsledky umožňují:

1. lépe porozumět tkáňově specifickému dopadu vybraných mtDNA mutací na funkce mitochondrií v autoptických tkáních

2. lépe porozumět souvislostem mezi mitochondriální ultrastrukturou a funkcemi u vybraných mitochondriálních a lysosomálních střádacích chorob na úrovni kultivovaných fibroblastů

1. INTRODUCTION

1.1 Mitochondria

Mitochondria are membrane organelle with wide range of live-important functions for eucaryotic cell. They are involved in ATP synthesis, oxidation of fatty acids, they also cooperate in apoptotic processes, in calcium homeostasis and they are the center of reactive oxygene radicals production. Furthermore, they are involved in porphyrine synthesis and take part in amino acid metabolism as well as in metabolisms of large spectrum of xenobiotics.

The mammalian organism fully depends on the oxidative phosphorylation system (OXPHOS) as on the main energy (ATP) producer of the cell. The majority of high energy source – ATP used for all active metabolic processes is produced in eucaryotic cells by oxidative phosphorylation (OXPHOS) in mitochondria. Disturbances of OXPHOS may be caused by mutations in either mitochondrial DNA (mtDNA) or nuclear DNA (nDNA), and environmental factors have also been shown to have important effects on OXPHOS. In human mitochondria contain a small circular DNA molecule (16 659bp) encoding 11mRNAs and 13 polypeptides that form, together with nuclear-encoded subunits, five inner-membrane OXPHOS complexes. For the translation of these 11 mRNAs (nine monocistronic and two bicistronic), mitochondria use its own separate translational system made of protein components encoded exclusively by nuclear genes and RNA components encoded by the mitochondrial genome (two ribosomal RNA genes and 22 transfer RNA (tRNA) genes). Mitochondrial translation is precisely controlled to meet tissue-specific demands for mtDNA-encoded structural subunits of the OXPHOS complexes (Antonicka et al., 2006). Although the basal components of the mitochondrial expression system are known, the mechanism of the system regulation in response to the metabolic needs of the cell is poorly understood (Taanman, 1999).

There are thousands of mtDNA in each cell, and pathogenic mutations of mtDNA may be present in all of mtDNA molecules or only in some of them. As a result, cells and tissues may harbor both normal (wild-type) and mutant mtDNA, a situation known as heteroplasmy. Therefore, in most cases, the phenotypic manifestation of the genetic defect occurs only when a threshold level is exceeded (phenotypic threshold effect). It has been shown that it is possible to inhibit considerably the activity of respiratory chain complexes, up to a critical value, without affecting the rate of mitochondrial respiration or ATP synthesis (biochemical

threshold effect) (Rossignol et al., 2003). The threshold for biochemical expression in cultured cells may be up to 95% for tRNA mutations (Bakker et al., 2000) and around 60% for mtDNA deletions (Hayashi et al., 1991). The degree of organ dysfunction will also depend on tissue's energy requirement; for instance, brain and muscle are dependent on oxidative phosphorylation, and neurological disorders and myopathy are common features of mtDNA mutations, whereas skin and other organs are often without pathological phenotype.

1.2 Mitochondrial structure

Mitochondria are localized in the cytoplasm of eukaryotic cells and they exist as a dynamic network that often changes shape and subcellular distribution. The number and morphology of mitochondria within the cell are controlled by precisely regulated rates of organelle fusion and fission (Bereiter-Hahn & Voth, 1994). The inner and outer membranes create two separate compartments and surround mitochondrial matrix. The inner membrane is folded into numerous cristae, which greatly increase its total surface area containing all respiratory chain complexes, ATP synthase, and various transport proteins. Mitochondria in rat and human hepatocytes as well as in human skin fibroblasts (HSF) grown in culture contain tubular cristae observed by high-resolution scanning electron microscopy. The fibroblast mitochondria were proved to be long and branched as compared to those in liver which were spherical in shape (Lea et al., 1994).

1.3 Mitochondrial disorders selected as a point of interest

Mitochondrial disorders caused by mtDNA mutations range from relatively mild, late-onset manifestation (e.g., sensorineural hearing loss, progressive external ophthalmoplegia, or ocular myopathy) to devastating and frequently fatal syndromes, such as MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like seizures) or MERRF syndrome (myoclonus epilepsy and ragged-red fibers). Furthermore, several lines of indirect evidence suggest that mitochondrial dysfunction also may play a role in common disorders such as diabetes mellitus or neurodegeneration (Schon & Manfredi, 2003). Despite improved characterization of the genetic defects that lead to mitochondrial disorders, the pathogenic mechanisms of these diseases are mostly very little understood. An electron microscopic observation of mitochondrial ultrastructure together with biochemical and genetic studies provide valuable insight into many aspects of mitochondrial biology and pathology.

We focused on characterization of fibroblast cell lines and tissues harbouring mutations in mitochondrial tRNA (3243A>G, prevalent for MELAS syndrome, 8344A>G, prevalent for MERRF syndrome, 8363G>A asociated with Leigh syndrome). Mutation in mtDNA gene for *ATP6* subunit (8993T>G) in mitochondrial F_0F_1 ATP synthase has been observed only on level of cultured human skin fibroblasts as well as impact of *SCO2* mutation on selected mitochondrial function.

1.4 Lysosomal storage diseases

Lysosomal storage diseases (LSDs) comprise more than 40 genetic disorders with cytoplasmatic accumulation of lipids, proteins and/or mucopolysaccharides. Some of LSDs are caused by inactivating mutations in lysosomal hydrolytic enzymes or by mutations that affect their delivery to lysosome. Other LSDs involve impaired export of hydrolysis products (Mach, 2002).

However, it is not clear if these substrates themselves are the primary mediators of toxicity. Indeed, the biological pathways from lysosomal enzyme deficiency to cellular dysfunction are still largely unknown. Interestingly, despite the great structural diversity of the accumulating substrates in the different LSDs, these disorders share many phenotypic similarities suggesting the presence of common pathogenetic mechanisms.

Postmitotic cells such as neurons and mononuclear phagocytes rich in lysosomes are most often affected by the accumulation of undegraded material. Cell death is well documented in parts of the brain and in other cells of LSD patients as well as in animal models. Although little is known about mechanisms by which death pathways are activated in these diseases, while not all cells exhibiting increased storage material are affected by cell death.

1.5 Autophagy

Lysosomes are essential for maturation and completion of autophagy-initiated protein and organelle degradation. Moreover, the accumulation of effete mitochondria has been documented in postmitotic cells whose lysosomal function is suppressed or in aging cells with lipofuscin accumulation (Terman et al., 2006).

Most LSDs involve impaired cognitive and motor functions. Loss of neurons is well documented in LSD patients and mouse models (Hara et al., 2006) supporting the concept that the nervous system is particulary susceptible to altered lysosomal functions.

It was suggested that lysosomal deficiencies in LSDs inhibit autophagic maturation, and lead to a autophagic stress condition. The resulting accumulation of dysfunctional mitochondria showing impaired Ca^{2+} buffering increases the vulnerability of the cells to pro-apoptotic signals (Kiselyov et al., 2007; Settembre et al., 2008)

Not only in LSDs but also in mitochondrial disorders, autophagy process takes part on pathogenesis. It was observed in fibroblasts culture harbouring 3243A>G mutation with high heteroplasmy loud (Gu et al., 2004) as well as in coenzyme Q deficiency in very recent study (Rodriguez-Hernandez et al., 2009).

Our point of interest is focused on the group of LSDs previously connected with mitochondrial dysfunction on biochemical level (Fabry disease (Lucke et al., 2004), Gaucher and Niemann Pick A disease (Strasberg, 1986)) and a group without described mitochondrial abnormalities (α-mannosidosis and metachromatic leucodystrophy).

Changes in autophagy has been also studied in selected group of mitochondrial disorders.

2. AIMS OF STUDY

Mitochondrial disorders represent a group of heterogenous diseases with clinical onset from newborn age till adulthood, mostly presented with very severe courses. The mammalian organism is fully dependent on mitochondrial oxidative phosphorylation system as the major energy producer of the cell. Therefore the mitochondrial disorders influence mainly high energy demanded tissues such is brain, heart or muscles. Similar phenotype is observed in many lysosomal storage disorders as well.

Despite of the expanding knowledge of molecular basis of mitochondrial and lysosomal disorders, it is still difficult to explain exact pathogenesis of the disease as well as prognosis for patients.

Mitochondrial functions affect more than just energy production; they contribute in initiation of apoptosis, in cellular calcium homeostasis and in production of reactive oxygene species. Disturbed mitochondria become a goal of autophagy mediated by the lysosomal compartement.

Specific aims:

1. To analyze tissue specific impact of selected mtDNA mutations on mitochondrial function in autoptic tissues

2. To analyze relations between mitochondrial ultrastructure and a function in selected mitochondrial and lysosomal storage disorders on the level of cultured fibroblasts.

3. MATERIAL AND METHODS

3.1 Ethics

This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and was approved by the Committee of Medical Ethics of the 1st Faculty of Medicine, Charles University and General Faculty Hospital, Prague. Informed parental consent, in accordance with the guidelines of the 1st Faculty of Medicine, Charles University, was obtained for all autopsies and skin biopsies.

3.2 Material

Autoptic tissues with selected mtDNA mutation were available from patients with mtDNA mutations: 8363G>A (skeletal muscle, heart muscle, brain tissue, liver tissue), 3243A>G (skeletal muscle, heart muscle, brain tissue, liver tissue), 8344A>G (skeletal muscle). Muscle biopsy was available from patients with mtDNA mutation 8363G>A.

Human skin fibroblasts were derived from patients harbouring:

- mtDNA mutation: MELAS 3243A>G (2 lines), MERRF 8344A>G (2 lines), NARP 8993T>G (1line) and Leigh syndrom 8363G>A
- LSDs: Fabry disease (5 lines), α - mannosidosis (3 lines), Niemann - Pick disease A (1 line), Gaucher disease (3 lines) and metachromatic leucodystrofy (1 line).

Up to 20 human fibroblast cell lines without any known mitochondrial or lysosomal storage diseases were used as controls.

3.3 Tissue culture

The cultures of fibroblasts were established from skin biopsy after informed parental consent. Fibroblasts were grown in the Quantum 333 medium (PAA, Austria) at 37°C in humid atmosphere containing 5% CO_2.

Inverted microscope Nikon Diaphot 200 was used for the imaging of cellular morphology in the phase contrast as well as for the analysis of mitochondrial reticulum visualized by MitoTracker green (MTG, Molecular Probes, USA) (1μM).

3.4 Activities of respiratory chain complexes

The activities of the mitochondrial enzymes NADH:CoQ reductase (complex I), succinate:CoQ reductase (complex II), cytochrome c oxidase (COX, complex IV), NADH:cytochrome *c* reductase (complex I+III), succinate:cytochrome *c* reductase (complex II+III) and citrate synthase (serving as the control enzyme) were measured spectrophotometrically at 37 °C in cultured fibroblasts by modified methods according to Rustin et al. The activities of COX were analyzed three times in doublets and they are expressed as mean ± SD, other complexes were measured in doublets.

3.5 Detection of mitochondrial mass, mitochondrial potential and production of reactive oxygen species (ROS)

Human skin fibroblasts were grown in 25 cm^2 cultivation flasks until approx. 90% of cell confluence was reached. Then, they were exposed to final concentration of 1 μM MitoTracker Green (Molecular Probes, USA) for mitochondrial mass measurement. Aliquots of cells were incubated with 20 nM TMRM (tetramethylrhodamine methyl ester) for detection of mitochondrial potential and 10 mM 2',7'-dichlorodihydro-fluorescein-diacetate (H2DCF-DA, Molecular Probes, USA) for measurement of ROS in cultivated medium in 37°C in humid atmosphere and 5% of CO_2 for 30 min. Cells were trypsinized, washed twice with PBS and transferred immediately to a tube on ice for flow cytometric analysis.

3.6 Flow cytometric analysis

FACS Calibur flow cytometer (BD, San Jose, CA USA) equipped with argon laser (488-nm) was used for the flow-cytometric analysis. Forward and side scatters were used to establish size gates and exclude debris from the analysis. Intensity of emitted fluorescence was detected using bandpass filters: 530 ± 15 nm (FITC) and 585 ± 21 nm (PE/PI), FL1 and FL2 channels, respectively. In each measurement, a minimum of 10 000 cells were analyzed. Data were acquired and analyzed using the Cell Quest software (BD, San Jose, CA USA). A relative change in the mean fluorescence intensity was calculated as the ratio between mean fluorescence intensity in the channel of the treated cells and that of the control cells.

3.7 Ultrastructure analysis

Cells were fixed based on Luft's modified method in PBS containing 2% potassium permanganate for 15 min, washed with PBS and dehydrated with an ethanol series. They were

then embedded in Durcupan Epon, sectioned by microtome Ultracut Reichert to thickness ranging from 600-900Å, stained with lead citrate and uranyl acetate (Luft, 1956). Transmission electron microscope (JEOL, JEM-1200EX) was used for imaging.

3.8 Morphometry

Modified Nonomura method was used (Nunomura et al., 2001): Micrographs of cultivated fibroblasts (derived from four controls cell lines and three patients cell lines) were taken at magnification of 5 000x and additionally at 20 000x. Five to ten sections were examined in each cell line. Intact mitochondria, swelled mitochondria containing cristae, swelled mitochondria without cristae was identified and counted. Percentage rate of each morphological group was calculated for each section. Differences between groups were evaluated by Mann-Whitney test.

3.9 Electrophoresis and immunobloting analysis

Tricine SDS/PAGE was carried out under standard conditions with 12% polyacrylamide, 0.1% (w/v) SDS and 5.5 M urea gels. Mitochondrial fractions were dissociated in 50 mM Tris/HCl (pH=6.8), 12% (v/v) glycerol, 4% SDS, 2% (v/v) 2-mercaptoethanol and 0.01% (w/v) Bromophenol Blue for 30 min at 37 °C. Approx. 10 μg of protein was loaded for each lane. Proteins were electroblotted from the gels on to Immobilon™-P PVDF membranes (Millipore) using semi-dry transfer for 1 h at a constant current of 0.8 mA/cm2. Immunodetection using monoclonal antibodies against structural subunits of respiratory chain complexes and ATP synthase (Mitosciences, USA) was performed according (Stiburek et al., 2005).

3.10 Fluorescence measurement of cytosolic Ca^{2+}

The cells grown on glass cover slips were washed in ECS (160mM NaCl, 2,5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Hepes-Na, 10 mM glucose; pH=7.4). After the washing, the cells were loaded with 1 μM Fura-2 acetoxymethyl ester (Fura-2/AM) for 60 min at 25 °C, in the dark, rinsed with and kept in ECS for 30 minutes prior to fluorescence measurements at 25°C. Ratiometric imaging was performed using Olympus microscope IX 50, based microspectrofluorimeter (Visitron Systems, Puchheim, Germany), equipped with cooled digital CCD camera (MicroMAX RTE/CCD -512EFT, Princeton Instruments, Monmouth Junction, NJ, USA). Fluorescence of Fura-2 was excited at wavelengths 340 and

380 nm switched by Polychrome II (Till Photonics, Planegg, Germany) illumination device. Emitted light at λ>420 nm (filter U-MWU) was recorded every 2 s, integration time for each wavelength was 300 ms. Software MetaFluor 7.0 (Universal Imaging Corp. West Chester, PA, USA) was used to control synchronization of excitation and data acquisition and for the visualization of the relative calcium concentration based on the ratiometric measurement.

3.11 Immunocytochemistry

Immunocytochemistry was performed according Capaldi et al. by usage of specific primary antibodies against cytochrome c oxidase (subunit II and Va) and porine (Mitosciences) and secondary antibodies (Alexa Fluor 488 and 564) (Capaldi et al., 2004).

3.12 Polarography

Muscle fibers were separated mechanically and oxygen consumption by saponin-skinned muscle fibers was determined using multiple substrate inhibitor titration as described previously (Wenchich et al., 2003).

3.13 Semiquantitative RT-PCR

Total RNA was isolated using the RNeasy kit (Qiagen). First-strand cDNA was synthesized using 1 μg of total RNA (DNase-treated) in a 20 μl reverse transcriptase reaction mixture and the requested part of cDNA was amplified using specific primers.

4. RESULTS AND DISCUSSION

Ad1: To analyze tissue specific impact of selected mtDNA mutations on mitochondrial function in autoptic tissues

4.1 The impact of mitochondrial tRNA mutations on the amount of ATP synthase differs in the brain compared to other tissues

In the 8363G>A skeletal muscle sample obtained at autopsy, profoundly decreased levels of complex I (5% of control) and IV (< 10% of control) were detected. The sample also revealed a diminished amount of complex V holoenzyme (35% of control), along with accumulated sub-complexes, most likely V* (F1-ATPase with several c-subunits) and F1-ATPase.

A very similar pattern, although less severe, was found in the 8344A>G skeletal muscle sample. The amount of complex I was reduced to approximately 25% of control, the level of complex IV holoenzyme was < 15% of control, and the holoenzyme level of complex V was decreased to 60% of the control value. Immunodetection of complex V further showed sub-complexes similar to those observed in the 8363G>A sample.

In 3243A>G skeletal muscle, relative levels of OXPHOS holoenzymes were normal, except for a decrease in the content of complex I to 30% of control and complex IV to 60% of control.

Only the 8363G>A and 3243A>G samples of heart, frontal cortex, and liver tissue were available for comparison of the impact of mtDNA mutations on the system of oxidative phosphorylation in skeletal muscle and other tissues. The 8363G>A heart sample showed the same considerable reduction in complexes I (5% of control) and IV (< 10% of control) content as skeletal muscle, but despite the greater reduction of complex V holoenzyme (15% of control), there was a significantly lower accumulation of F_1-ATPase The 3243A>G heart sample had a pronounced reduction of complex I (20% of control), similar to skeletal muscle.

In the 8363G>A frontal cortex, the assembly of complexes I and IV were less affected than in the above-mentioned tissues. The amount of complex I was decreased to 40% of control, and the holoenzyme level of complex IV was reduced to 50% of control. Conversely, complex V (<20% of control) appeared to be the most severely affected member of the OXPHOS system. The decrease was more substantial than in skeletal muscle; nevertheless, no detectable sub-complexes could be found. The 3243A>G frontal cortex sample showed a

dramatic reduction of complex V to below the detection limits of the method, and of complex I to 10% of the control value. Probing of the immunoblots with an anti-COX2 antibody showed a reduction of complex IV to 20% of control, as well as the presence of a high molecular weight sub-complex. Due to the unexpected OXPHOS deficiency pattern observed in brain tissue, particularly in the 3243A>G patient, the immunoblotting was also performed on the frontal cortex sample from another 3243A>G patient. In the only available sample with a 65% level of heteroplasmy, the immunoblotting analysis revealed normal levels of OXPHOS complexes in comparison to control.

Despite the highest level of heteroplasmy, the 8363G>A liver sample only showed an isolated deficiency of complex I (40% of control). Steady-state levels of OXPHOS complexes in the 3243A>G liver mitochondria were comparable to control.

In 8363G>A skeletal muscle, the level of free S1 sub-complex was found to be below the detection limit of the method. This probably reflects the limiting character of the COX1 subunit in the holoenzyme assembly. In 3243A>G frontal cortex, significantly increased levels of all known complex IV assembly intermediates were observed, including free apoCOX1 (S1 sub-complex), apoCOX2, and apoCOX5A. The high molecular weight band just below the COX holoenzyme, which was detected with anti-COX2 antibody, very likely represents the S3 assembly intermediate.

Consistent with immunoblotting results, the spectrophotometry revealed lower activity ratios for complex I (I/II and I/CS) in the isolated muscle mitochondria of all three patients, and a severe deficiency of complex IV in 8363G>A and 8344A>G isolated muscle mitochondria. The COX/SQR (IV/II) and COX/CS (IV/CS) ratios in the 3243A>G patient were just below the control range.

The functional consequences of the 8363G>A mutation in comparison to the 8344A>G mutation were analysed by high-resolution oxygraphy of the patients´ skeletal muscle fibers permeabilized by a low concentration saponin treatment. In both samples, a decrease of ADP-stimulated oxygen consumption compared to control fibers was observed using pyruvate as a substrate, and an increase was found after succinate addition. A pronounced reduction in ADP-stimulated respiration was found after pyruvate (33% of the mean control value) as well as after glutamate (32% of the mean control value) additions in 8363G>A muscle fibers. Indeed, ADP-stimulated respiration after succinate treatment increased to 158% of the mean control value. In 8344A>G muscle fibers, ADP-stimulated respiration after pyruvate addition was 70% of the mean control value, but after glutamate, it was within the reference range. Similarly to 8363G>A muscle fibers, the ADP-stimulated

respiration after succinate addition increased to 222% of the mean control value in the 8344A>G sample. The absolute oxygen consumption after ascorbate + TMPD treatment was, in both samples, within control levels; however, normalization of the data to the level of respiration after succinate addition revealed decreased ratios after treatment with all of the substrates (Fornuskova et al., 2008).

In the skeletal muscle the lowest level of heteroplasmy but the most severe OXPHOS defect suggests a more profound impact of the 8363G>A mutation on the translational system than that of the 8344A>G mutation. Concerning the 3243A>G mutation, the relatively proportional levels of heteroplasmy in 8344A>G and 3243A>G skeletal muscles, the more significant decrease in the content of complex I, the severe decrease in the amount of complex IV, and the slightly lower level of complex V all indicate a less pronounced impact of the 3243A>G mutation on mitochondrial translation in this tissue. However, the different nuclear backgrounds (Feuermann et al., 2003), distributions of heteroplasmy levels in cells and mitochondria (Durham et al., 2007), and environmental factors (Levy, 2007) which have been shown to influence expression of mitochondrial respiratory insufficiency, prevent the reduction of these results to any simple quantitative trait. The frequency of UUR (Leu) codons in mitochondrially-translated subunits of OXPHOS implies decreased steady-state levels of complex I subunits, namely ND6, ND3, ND2 and ND5 (14 – 9 UURs) in 3243A>G mitochondria. In 8363G>A and 8344A>G mitochondria, the distribution of AAR (Lys) codons anticipates diminished levels of ND5, ND2, ND4 and COX1 (21 - 10 AARs). Moreover, all these subunits contain two X/Lys/Lys/X motifs, or one X/Lys/Lys/X and one Lys/X/Lys motif (http://www.mitomap.org), which are apparently strong stalling points for the ribosome.

The particular abundance and distribution of codons in mitochondrially-translated subunits of respiratory chain complexes appears to be a plausible explanation for the isolated defect of complex I in the patient with a mutation in mt-tRNA$^{Leu(UUR)}$, along with the combined deficiency of complexes I and IV in patients with mutations in mt-tRNA^{Lys}.

Furthermore, the complete absence of unassembled apoCOX1 in the skeletal muscle of the 8363G>A patient, as revealed by BN/SDS/PAGE immunoblotting, conforms to the limiting character of the COX1 subunit in holoenzyme assembly. On the contrary, the OXPHOS deficiency patterns found in the frontal cortex mitochondria of the 8363G>A and 3243A>G patients could suggest, similarly to (stiburek et al., 2005), a specific character for brain OXPHOS. First, in both frontal cortex samples, the decrease in the content of complex V was more profound than in that of complexes I and IV. Second, despite such a pronounced

defect of complex V, no sub-complexes similar to those observed in skeletal muscle and heart could be detected, even when the immunoblot exposure was prolonged (data not shown). Unfortunately, no 8344A>G frontal cortex specimen was available for the analysis. However, a selectively decreased expression of COX2 subunit was previously reported in frontal cortex and cerebellum of a MERRF patient (Sparaco et al., 1995). F_1-ATPase, which was observed in heart, was found along with a sub-complex denoted V* in skeletal muscle. The sub-complex V* is likely composed of F_1-ATPase and several c-Fo subunits. These sub-complexes were described previously in 8993T>G mitochondria (Carrozzo et al., 2006), ρ^0 cells (Carrozzo et al., 2006), and in cells with inhibited mitochondrial translation (Nijtmans et al., 1995). The observed steadystate levels of OXPHOS complexes suggest that the brain ATP synthase is most sensitive to disturbances of the mitochondrial translational system caused by the studied mt-tRNA mutations. Such a tissue-specific impact of mt-tRNA mutations with comparable tissue heteroplasmy is likely to result from tissue-specific variations in the nature of mitochondria. Indeed, it was shown that the brain, liver and kidney OXPHOS system is mainly controlled at the phosphorylation level by ATP synthase and a phosphate carrier, in contrast to the muscle and heart, where it is essentially controlled at the level of the respiratory chain (Rossignol et al., 2000). On the other hand, instead of diminished energy provisions, an insufficient discharge of mitochondrial membrane potential leading to reactive oxygen species (ROS) production was proposed as the underlying pathogenic mechanism of ATP synthase deficiency (Houstek et al., 2004). Accordingly, the complete lack of complex V from 3243A>G frontal cortex may be, apart from the translational defect, responsible for the unusual assembly pattern of COX in this sample. Indeed, it was shown in yeast that (i) cells deficient in ATP synthase have a severe reduction of COX holoenzyme (Rak et al., 2007) and (ii) no decrease in COX synthesis is observed in uncoupled ATP synthase mutants, where the maintenance of mitochondrial potential is severely compromised by a massive proton leak through the Fo sector (Houstek et al., 2004). Although the COX1 subunit was shown to be a key regulatory target for COX reduction in yeast cells (Rak et al., 2007) some other mechanism is likely to be involved in the hindered assembly of COX in the 3243A>G frontal cortex mitochondria, since this sample had high accumulated levels of all three mitochondrially encoded subunits, either free or partially assembled.

Although it is necessary to analyze considerably more samples with high levels of heteroplasmy (such samples are difficult to obtain), these data show new effects of mt tRNA mutations on the brain which differ substantially from those described for skeletal muscle, heart, and liver tissues.

Ad 2: to **analyze connection between mitochondrial ultrastructure and function in selected mitochondrial and lysosomal storage disorders on level of cultured fibroblasts.**

4.2 Mitochondrial ultrastructure and function in selected mitochondrial and lysosomal storage disorders on level of cultivated fibroblasts

Ultrastructural analysis of cultivated fibroblasts of our patients with mtDNA point mutations revealed heterogeneous mixture of variously changed mitochondria with unusual, rare cristae. This heterogenity in size and shapes of mitochondria in a single cell suggests the presence of different levels of heteroplasmy within the individual cell (intracellular heteroplasmy, even further intramitochondrial: a single mitochondrion can harbor several copies of mtDNA, in that case both: normal and mutant mtDNAs). Intercellular heterogenity in mitochondrial morphology of heteroplasmic cells carrying the mtDNA mutation 3243A>G were described by Bakker et al. (Baker et al., 2001). They observed that the number of defective cells increased with increasing mutation load. The cristae of our patients' mitochondria with mtDNA mutation 3243A>G, 8344A>G, and 8993T>G were poorly developed, occurred sparsely or were absent. The control samples' mitochondria had numerous long cristae, often like septa, and filamentous morphologies of mitochondria were far more prevalent than the oval ones (Brantova et al., 2006) (Fig.1)

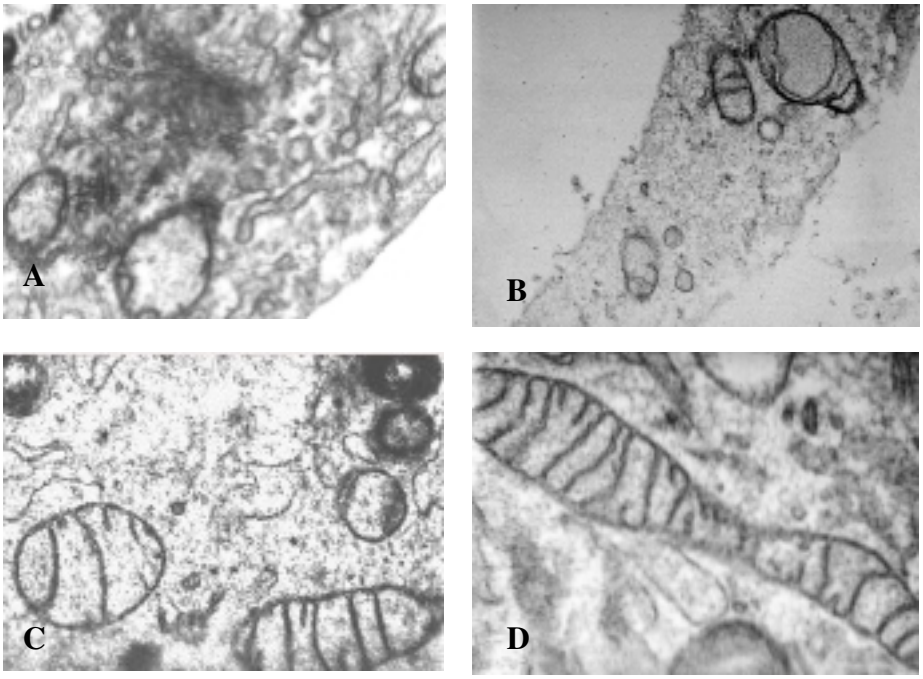

Fig.1: Effete mitochondria in cultivated human skin fibroblasts derived from patients with mitochondrial disease (A-C) and derived from control cell line (D).

Very similar mitochondrial abnormalities to mitochondrial disorders harbouring cells were observed in all examined LSDs cell lines. In addition, dilated endoplasmatic reticulum (ER) was obvious in fibroblasts derived form patients with α-mannosidosis.

Moreover, in both – mitochondrial disorders and LSDs cell lines, mitochondria with damaged cristae had a swollen and bubble-like structure, a diagnostic marker related with injured or missing cristae. Similar observations of the mitochondrial morphological changes in fibroblasts were also described by other autors (Procaccio et al., 1999).

The correlation of the ultrastructural abnormalities of cristae with the impact on their function is based on location of oxidative phosphorylation. Gilkerson et al. (Gilkerson et al., 2003) assumes that around 94% of both respiratory chain complex III and the ATP synthase are located in the cristal membrane, and only around 6% of either is in the inner boundary membrane; both complexes exist at a 2.2- to 2.6-fold higher concentration in the cristal membrane. Further, related data showed the ATP synthase to be involved in the process of generating mitochondrial cristae morphology (Paumard et al., 2002). Convincing argument for topology of the mitochondrial inner membrane and bioenergetic implications brings electron tomography together with computer modeling studies using the Virtual Cell program. The shape of the inner membrane can influence OXPHOS function. Simulation indicates that narrow cristae junctions restrict diffusion between intracristal and external compartments, causing depletion of ADP and decreased ATP output inside the cristae (Mannella et al., 2001).

All this evidence may explain how mitochondrial morphology, function, and bioenergetics are linked together. Nevertheless, the primary cause of all documented pathology lies in genetic defects proved by mtDNA analysis. The onset and severity of clinical manifestations depend on a delicate balance between the energy supply (determined in part by the proportion of mutant and normal mtDNA) and oxidative demands of different organ systems.

4.2.1 Activities of respiratory chain enzymes

Fibroblasts cell lines harbouring mutations in mtDNA did not reveal any profound changes in activities of respiratory chain complexes compare to the controls except line with mtDNA mutation 8363G>A, where the activity of cytochrome c oxidase (COX) was under detection limit of used methods. Boundary activity of COX was detected in one patient with high heteroplasmy loud of mtDNA mutation 3243A>G.

Majority of examined cell lines derived from patients with LSDs revealed disbalance in activities of respiratory chain, some of them with tendency to increased activities of COX or complex I. The most profound changes were detected in cell cultures derived from patients with α-mannosidosis, where the activity of respiratory chain complexes and control enzyme citrate synthase revealed significantly increased activity of complex IV in all studied cell lines compare to controls (p=0,05), the ratio between complex IV and CS was increased as well. In addition, the activity of complex I or complex I+III was at the upper border of the reference range or increased compare to the controls. It resulted in increased ratio between complex I or I+III and CS in each studied fibroblasts. The activities of respiratory chain complex II were in reference range and the activities of CS were slightly decreased in one of patients. (Brantova et al., 2009).

Mild decreased of COX activity was observed in cell lines derived from patients with Gaucher disease and metachromatic leucodystrophy, however, after normalization to citrate synthase, the values reached reference range.

Contrary to our results, declined activities of all respiratory complexes except complex II were described in cultivated fibroblasts from patients with Fabry disease (Lucke et al., 2004). Therefore we examined influence of elevated level of storage material (globotriosylceramide, GB3) added to cultivated medium on respiratory chain and we did not detect any declination in enzyme activities. There is a possibility, that increased load GB3 was not sufficient to cross a treshold level to reach the described phenotype.

4.3 Mitochondrial mass and superoxide production

Cultivated fibroblasts with mtDNA mutation 3243A>G a 8344A>G revealed tendency to increase mitochondrial mass as well as level of mitochondrial superoxide contrary to controls. Although previous study did not detect any changes in mitochondrial mass (James et al., 1996), our observation is in accordance with tight connection between elevated oxidative stress and mitochondrial mass (Lee et al., 2000).

Elevating of oxidative stress in fibroblasts from patients with SCO2 and NARP syndrome, we did not observe increased mitochondrial mass. Explanation may be due to elevated rate of autophagy.

Production of mitochondrial superoxides was detected in all fibroblasts cell lines derived from patients with LSDs except Fabry disease. Significant change in mitochondrial mass was observed only in cultures derived from patients with α-mannosidosis, where we observed declination in mitochondrial mass.

Elevated level of mitochondrial superoxide production could advert to accumulation of effete mitochondria in cells, which is in accordance with elelectron microscopy observation.

High level of oxidative stress was previously observed in neurons derived from patients with Gaucher disease (Deganuto et al., 2007). In our study, we focused on mitochondria and we could complete the information about mitochondrial participation on oxidative stress in LSDs.

Contrary to our results, high level of reactive oxygene species in cultivated endothelial cells obtained from patients with Fabry disease was detected, however only when the cells were cultured in medium supplement with patients serum (James et al., 1996). It could be one of the reasons for normal findings in our fibroblasts cell lines. Another explenation may be non-mitochondrial origin of oxidative stress in cells.

4.3.1 Mitochondrial calcium homeostasis in HSF derived from selected mitochondrial and lysosomal storage disorders

The amount of calcium retained in mitochondria was monitored by usage of cytoplasmatic probe Fura 2AM, further Bradykinine and FFCP.

During release of calcium from ER after Bradykinin treatment, part of it is absorbed by mitochondria. FCCP releases mitochondrial calcium to cytoplasm.

Our study showed profound changes in mitochondrial calcium amount in cells derived from patients with MELAS, MERRF and SCO2 syndromes as well as in fibroblasts from Gaucher and Niemann Pick A disease. Only mild declination in mitochondrial calcium amount was detected in cell culture from patients with NARP syndrome; fibroblasts with α - mannosidosis and Fabry disease remained in normal.

Disturbed calcium homeostasis was previously documented in large scale of mitochondrial disorders. Rizzuto et al. described different answers to stimulation of calcium metabolism in fibroblasts harbouring mtDNA mutations 8356T>C and 8993T>G with impact on cell ATP production (Brini et al., 1999). Elevated level of cytoplasmatic calcium was observed in mtDNA mutations 3243A>G a 8344A>G. It could be related to disturbed mitochondrial ability to buffer cellular calcium (Moudy et al., 1995).

Pathology of Gaucher (Lloyd-Evans et al., 2003) and Niemann Pick A disease (Ginzburg & Futerman, 2005) is connected to impaired calcium homeostasis. However calcium disbalance has never been connected to disturbance of mitochondria.

Elevated calcium concentration in cell cytoplasm implicates higher oxidative stress and higher intramitochondrial amount of calcium activates respiratory chain. It is in concordance with our results.

4.3.2 Autophagy

Autophagy has been monitored during cell starvation as the presence of LC3I and LC3 II after immunoblot detection.

Tendency to elevated autophagy rate was observed in fibroblasts derived from patients with MERRF and SCO2 syndrome.

Effect of autophagy on elimination of effete mitochondria was documented previosly in cell culture derived from patients with MELAS syndrom (Gu et al., 2004) as well as in coenzyme Q deficiency (Rodriguez- Hernandes et al., 2009).

No changes of autophagy rate was observed in fibroblast derived from selected LSDs, except Fabry disease after higher cultivation in medium with storage material, GB3. Mentioned cell line revealed blocking of autophagy on protein level. It was confirmed on transcriptional level by RT-PCR.

Based on our results, we conclude effect of autophagy on mitochondrial disorders and possible mechanisms of elimination of ROS production in condition of elevated autophagy.

The block of autophagy observed in one patients with increased intracellular storage could be more profound in other energy demanded disorders.

5. CONCLUSION

The results of our study improved the knowledge of exact pathogenesis in mitochondrial disorders and in LSDs disorders with secondary disturbed mitochondrial functions, especially in:

- better understanding of tissue specific impact of selected mtDNA mutations on mitochondrial function.

  Obtained data from autoptic tissues revealed new effects of mt tRNA mutations on the brain which differ substantially from those described for skeletal muscle, heart, and liver tissues.

- better understanding of relation between mitochondrial ultrastructure and function in selected mitochondrial and lysosomal storage disorders on the level of cultured fibroblasts.

  Based on our results, we conclude primary effect of mutations connected with mitochondria as well as secondary influence of LSDs on mitochondrial ultrastructure, respiratory chain activities, mitochondrial superoxide production, calcium disbalance and autophagy homeostasis.

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