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Sites and consequences of

mitochondrial ROS production

PhD Thesis

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I would like to dedicate the thesis to my parents.

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Contents

i

Abbreviations

AA	antimycin A
ADP	adenosine diphosphate
ANT	adenine nucleotide translocator
ATP	adenosine triphosphate
Atpaf1p, Atpaf2p	ATPase assembly proteins
BAT	brown adipose tissue
C I, II, III, IV	mitochondrial respiratory chain complexes I, II, III and IV respectively
cGPDH	cytosolar glycerophosphate dehydrogenase
CoA	coenzyme A
Q	coenzyme Q
COX	cytochrome c oxidase
CPEO	chronic progressive external ophtalmopelia
Cu/ZnSOD	copper and zinc superoxide dismutase
cyt	cytochrome
DHAP	dihydroxyacetone phosphate
EFG1	elongation factor G 1
EPR	electron paramagnetic resonance
ETF	electron-transferring flavoprotein
FADH$_2$	flavin adenine dinucleotide, reduced form
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FeS	iron – sulphur cluster
FH	fumarate hydrogenase
FMN	flavin mononucleotide
GP	glycerol-3-phosphate
Glu	glutamate
GPx	glutathione peroxidase
GTP	guanosine triphosphate
HIF	hypoxia-inducible transcription factor
HRP	horse-radish peroxidase
KGDH	α-ketoglutarate dehydrogenase
KSS	Kearns-Sayre syndrome
LHON	Leber's hereditary optic neuropathy
LRPPRC	leucine-rich PPR-motif containing

MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonical epilepsy and ragged red fibers
mGPDH	mitochondrial glycerophosphate dehydrogenase
MnSOD	manganese superoxide dismutase
MRPS16	mitochondrial ribosomal protein S16
mtDNA	mitochondrial deoxyribonucleic acid
Mxt	myxothiazol
NADH	nicotinamide adenine dinucleotide, reduced form
NARP	neurogenic muscle weakness, ataxia and retinitis pigmentosa
nDNA	nuclear deoxyribonucleic acid
OPA1	human dynamin-related GTPase, mediator of mitochondrial fusion
OXPHOS	oxidative phosphorylation
PDH	pyruvate dehydrogenase
pyr	pyruvate
QFR	menaquinol:fumarate oxidoreductase
ROS	reactive oxygen species
Rot	rotenone
RRF	ragged red fibers
rRNA	ribosomal ribonucleic acid
SDH	succinate dehydrogenase
TCA	tricarboxylic acid cycle
TMRM	tetramethylrhodamine methyl ester
TR/RXR	thyroid receptor / retinoid X receptor heterodimer
TRE	thyroid respone element
tRNA	transfer ribonucleic acid
UCP	uncoupling protein
VDAC	voltage-dependent anion channel
Δp	protonmotive force
ΔpH	gradient of protons
$\Delta\mu_H^+$	electrochemical proton gradient
$\Delta\Psi_m$	mitochondrial membrane potential

1 Introduction

1.1 Mitochondria and its structure

Mitochondria, as we all know them from the cartoons of eukaryotic cell structure, are small oval organelles 0.5 − 1 μm in diameter and several μm in length. But neither of these two facts is necessarily true, which illustrates our still limited knowledge about mitochondria. Their shape and size undergoes dynamic changes and in many cases they form a complex reticulum interacting with other organelles, such as endoplasmic reticulum.

According to endosymbiotic theory, mitochondria evolved from α-proteobacteria. (Gray et al., 1999). At least when viewed through the prism of antropic principle, the reason for such symbiosis was to provide the original cell with energy under aerobic conditions - almost 90% of energetic demands of mammalian organisms is covered by ATP generated by the oxidative phosphorylation (OXPHOS) process in mitochondria. But there is more than direct ATP production, mitochondria are involved in − regulation of cellular Ca^{2+} homeostasis, apoptosis and also generation of reactive oxygen species (ROS), phenomenon inherently connected to aerobic oxidation (Nicholls et al., 2000).

1.1.1 Structure of mitochondria

Mitochondria are surrounded by the inner and outer membranes that create two separate mitochondrial compartments: the internal matrix space and a narrow intermembrane space (Figure 1). The smooth outer membrane contains the proteins essential for import of nuclear-encoded mitochondrial proteins and the protein called porin, which forms aqueous channels, permeable to all molecules with molecular mass less than 5 kDa. The inner membrane is folded into numerous cristae, which greatly increases its total surface area. This membrane contains mainly proteins with three types of functions: the enzymes of the respiratory chain, ATP synthase complexes, and specific transport proteins that regulate the passage of metabolites and macromolecules across the inner mitochondrial membrane. Since the electrochemical proton gradient is generated across the inner membrane, it is important that the membrane phospholipid bilayer is impermeable to ions.
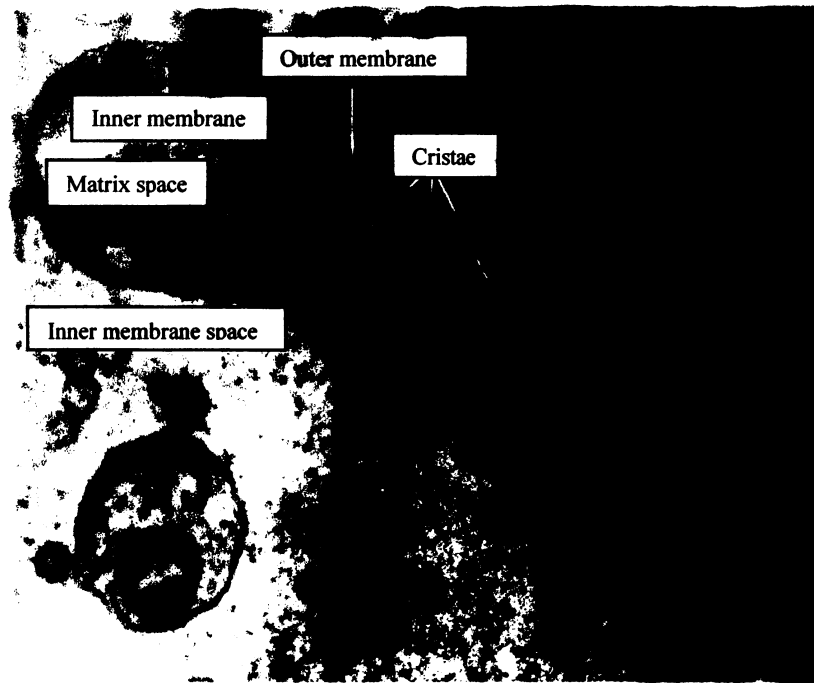
Figure 1: The basic structure of a mitochondrion as viewed by electron microscopy
Mitochondria are encircled by two membranes. The inner membrane folds into cristae, thus greatly increasing its surface. (Illingworth, 2002).

1.1.2 Electron transport sequence

The mitochondrial respiratory chain (Figure 2) transfers electrons form reduced cofactors (NADH, FADH$_2$) through a sequence of redox reactions to molecular oxygen. The redox potential span is 1.1V from NAD$^+$/NADH couple (-0.315 V) to the O$_2$/2H$_2$O couple (+0.815 V). Many of the respiratory chain reactions are reversible. To enable operation in both the forward and reverse reactions, it is necessary for the redox components to operate under conditions where both the oxidized and reduced forms exist at appreciable concentrations. Electrons can be fed to respiratory chain at several points.

The initial transfer of electrons from the soluble dehydrogenases of the citric acid cycle is filled by NAD$^+$/NADH couple which shuttles electrons to complex I (C I). It fulfils both important prerequisites for this function – mid-point potential in the region of -300 mV and high mobility. Most electrons pass into the respiratory chain in this way. On the other hand, couple of dehydrogenases catalyzes reactions with a mid-point potential close to 0 mV and therefore are not thermodynamically capable of NAD$^+$ reduction.
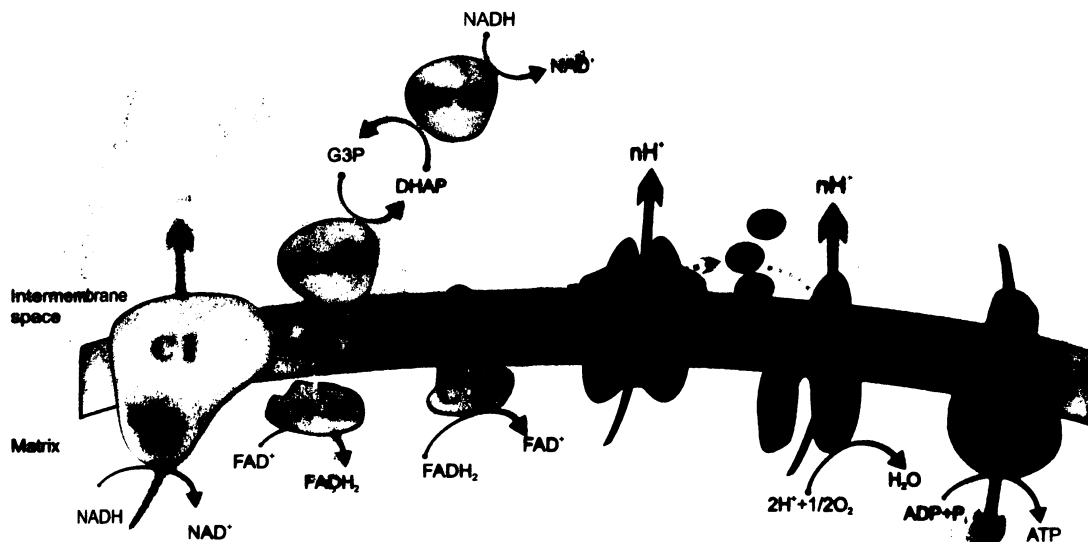
Figure 2: An overview of the mitochondrial respiratory chain. Yellow arrows represent transport of electrons, red arrows the coupled proton transport and the black ones reactions catalyzed by the respective enzyme complex. cGPDH / mGPDH – cytosolic / mitochondrial glycerophosphate dehydrogenase; ETF-OX - electron-transferring flavoprotein ubiquinone oxidoreductase; Q – coenzyme Q; cyt c - cytochrome c; G3P - glycerol-3-phosphate; DHAP - dihydroxyacetone phosphate; I, II, III, IV and V - complexes of respiratory chain. OXPHOS complexes are depicted in green, ATP synthase in red and mobile electron carriers (Q and cyt c) in orange. Note that mobile carriers are present in stoichiometric excess to OXPHOS complexes.

These dehydrogenases – namely succinate dehydrogenase (C II or SDH), mitochondrial glycerophosphate dehydrogenase (mGPDH) and acyl-CoA dehydrogenase – transfer electrons directly to Q pool at a potential close to 0 mV, independently on the $NAD^+/NADH$ couple. The third step is represented by complex III (C III) which transfers e^- from two-electron carrier coenzyme Q to cytochrome c, one-electron carrier. The electrons from cytochrome c are transferred by cytochrome c oxidase (C IV or COX) to O_2 which serves as terminal electron acceptor.

1.1.3 Proton transport and coupling

The energetically favorable flow of electrons through the respiratory chain is connected to the pumping of protons from the matrix into the intermembrane space (Figure 3). Three of the respiratory chain complexes posses proton translocating activity: C I, C III and C IV.

The movement of protons across the inner mitochondrial membrane has two major consequences (Gennis, 1989): it generates a pH gradient (ΔpH) and membrane potential ($\Delta\Psi_m$), as a consequence of the uncompensated net movement of positive ions (H^+).

Both components constitute an electrochemical proton gradient ($\Delta\mu_H^+$):

$$\Delta\mu_{H^+} = F\Delta\psi_m - 2.303\, RT\, \Delta pH$$

Usually it is expressed in millivolts as Δp – the protonmotive force as defined by P. Mitchell. (Mitchell, 1961):

$$\Delta p = \frac{\Delta\mu_{H^+}}{F} = \Delta\psi_m - 2.303\frac{RT}{F}\Delta pH$$

F, R, T are Faraday constant, gas constant and absolute temperature, respectively. Various studies on mammalian mitochondria revealed that the membrane potential (~170 mV) represents the main component of Δp in comparison with the pH gradient (~ 0.5 - 1 pH units, which corresponds to 30-60 mV). This is mainly due to the low electrical capacitance (~1 μF/cm^2) of the inner mitochondrial membrane (Nicholls et al., 1992).

The energy of protonmotive force is utilized by F_1F_0-ATP synthase (ATPase) to synthesize ATP (Figure 3). The ATP is synthesized intra-mitochondrially and then translocated to the cytosol in exchange for ADP by ADP/ATP translocator (ANT).

The coupling between RC proton translocation and ATP synthesis is not and might not be completely tight. First of all, transport of metabolites and ions into or out from the mitochondria also utilizes proton gradient. Furthermore there exists a family of mitochondrial uncoupling proteins (UCP1, UCP2, UCP3, UCP4) (Erlanson-Albertsson, 2002) which were proposed to regulate the level of mitochondrial membrane potential. This in turn may also influence extent of mitochondrial ROS production. Under circumstances, where mitochondrial ROS were proposed to play role in cellular redox signalling, this may further influence physiological response (see (Brand et al., 2005)). Perhaps the most specific function has UCP1 which is present in mitochondria of mammalian brown adipose tissue (Heaton et al., 1976). Here it shunts the proton gradient and therefore leads to heat production (Figure 3).
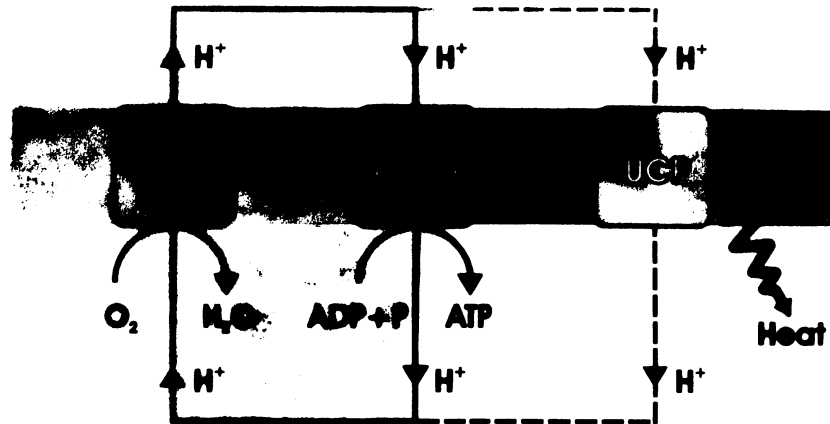
Figure 3: Coupling between respiratory chain and ATP synthesis. Protons are pumped by the respiratory chain (RC) across the inner mitochondrial membrane and proton gradient (negative inside) is formed. This gradient is then utilized by ATP synthase (ATPase) to synthesize ATP from ADP + P. The gradient may eventually be dissipated by the action of uncoupling proteins (UCP), which results in the production of heat.

1.2 Oxidative Phosphorylation Complexes

1.2.1 Complex I - NADH dehydrogenase

The complex I is the largest and most complicated of respiratory chain complexes. In mammalian mitochondria it is composed of at least 43 subunits and the approximate molecular weight is 800 kDa. Seven of these subunits are encoded by mitochondrial genome, the rest by nuclear (Attardi et al., 1986; Chomyn et al., 1985). According to electron microscopic data, CI is an L-shaped molecule, with larger integral membrane part and smaller arm protruding into mitochondrial matrix (Grigorieff, 1998). So far there is no crystal structure of mammalian complex I. Only the hydrophilic domain of the bacterial enzyme has recently been crystallized (Sazanov et al., 2006) (Figure 4).

The complex I contains several electron transporters, flavin mononucleotide (FMN), multiple Fe-S centres (also called iron sulphur clusters) and probably also bound coenzyme Q. The complex I contains two binuclear (2Fe-2S) clusters N1a and N1b and four tetranuclear (4Fe-4S) clusters N2, N3, N4 and N5 (Ohnishi, 1998).
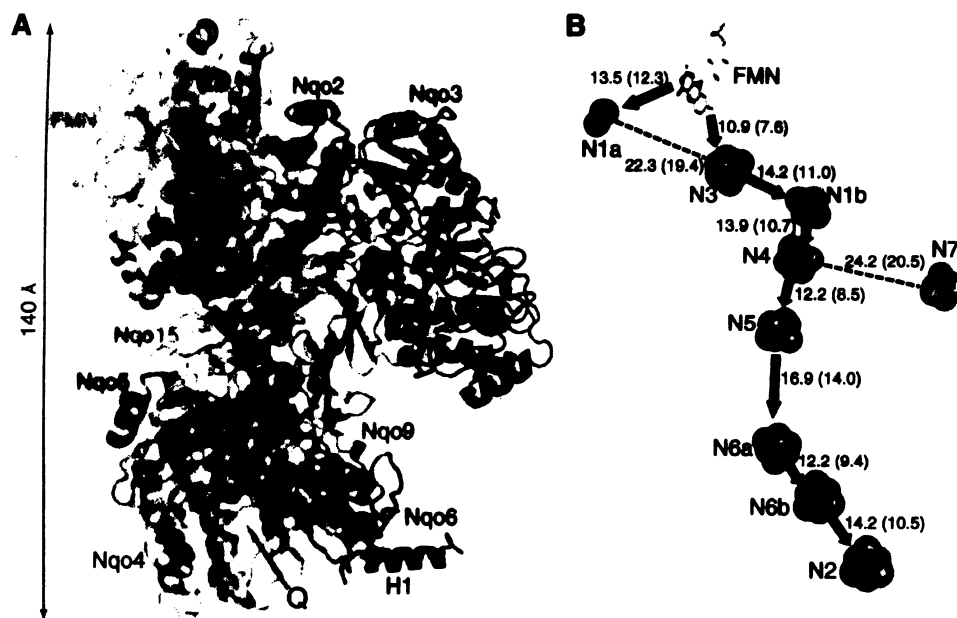
5

Figure 4: Structure of hydrophilic domain of complex I from *Thermus thermophilus.* (A) Side view, with the membrane arm likely to be beneath and extending to the right, in the direction of helix H1. Each subunit is coloured differently; FMN is shown as magenta spheres, metal sites as red spheres for Fe atoms and yellow spheres for S atoms. Proposed Q-binding site is indicated by an arrow. (B) Arrangement of redox centres and proposed electron transfer pathway based on edge to edge distances of redox centres (shown in parentheses). Cluster N1a is in subunit Nqo2; N3 and FMN in Nqo1; N1b, N4, N5, and N7 in Nqo3; N6a/b in Nqo9; and N2 in Nqo6. Cluster N7 is not part of the transfer pathway and is possibly an evolutionary remnant. Cluster N1a also does not lie on the direct pathway – it was proposed to play antioxidant role by scavenging electrons from FMN. Taken from (Sazanov et al., 2006)

Electrons are transferred from FMN to cluster N3 and then through a series of five isopotential clusters to the terminal cluster N2, located close to the Q-binding site. Based on crystal structure of the bacterial enzyme the full pathway was proposed to be: NADH → FMN → N3 → N1b → N4 → N5 → N6a → N6b → N2 → Q (Figure 4). Cluster N7 is too far from the other clusters to accept electrons effectively. Furthermore, this cluster is present only in bacterial enzyme and is therefore irrelevant for mammalian complex I.

Cluster N1a was proposed to play antioxidant function. The FMN, after reduction by NADH to $FMNH_2$, releases two electrons sequentially in two one-electron steps. Electrons can be transferred effectively to the nearest cluster N3 and along the cluster chain to membrane-embedded Q. However, under steady-state conditions are all Fe-S clusters in complex I almost fully reduced, as the rate-limiting step is likely the oxidation of N2 by Q (Kotlyar et al., 1990). When cluster N3 is reduced, electron transfer from

flavosemiquinone to cluster N1a becomes thermodynamically favourable. As soon as N3 is reoxidized during quinone reduction, electrons from N1a can proceed back via FMN into the main cluster chain. A pair of electrons from FMN may thus be donated nearly simultaneously to clusters N3 and N1a, and the flavosemiquinone radical may be short-lived. Furthermore, contrary to FMN, cluster N1a is shielded from the solvent. Therefore, such temporary storage of electrons by N1a is likely to minimize ROS production during physiological complex I activity (Figure 4).

The proton pumping machinery of complex I is represented by subunits ND1, ND5 and TYKY. TYKY subunit is special kind of ferredoxin which propels the proton translocating machinery formed by ND1 and ND5 (Albracht et al., 2000). The complex I translocates 4-5 protons per single electron pair transferred to coenzyme Q.

1.2.2 Complex II - succinate dehydrogenase

Mitochondrial complex II is a key membrane complex in the citric acid cycle that catalyzes oxidation of succinate to fumarate in the mitochondrial matrix. Succinate oxidation is coupled to reduction of ubiquinone to ubiquinol. Thus, this enzyme directly links citric acid cycle and oxidative phosphorylation. Succinate:ubiquinone oxidoreductase normally consists of a soluble catalytic heterodimer and an integral membrane region. The soluble part is made up of subunit A (Fp or SDHA) with covalently bound FAD and subunit B (Ip or SDHB) containing three iron-sulphur clusters: [2Fe-2S], [4Fe-4S] and [3Fe-4S] (Hagerhall, 1997). The integral membrane region anchoring the complex to the inner membrane consists of two transmembrane proteins: large cytochrome b binding protein (CybL or SDHC) and the small cytochrome b binding protein (CybS or SDHD) and contains one haem (Lemos et al., 2002). All subunits of complex II are encoded by nuclear genome. Unlike $E.$ $coli$ SDH, mitochondrial complex II seems to be active as a monomer and has a molecular weight of 120 kDa (Capaldi et al., 1977).

Recently both the structures of bacterial SDH (Yankovskaya et al., 2003) and mitochondrial complex II (Sun et al., 2005) were solved (Figure 5). These results offer important insights into mechanism of electron transfer and protection from ROS production at the level of C II.

According to the structural data it seems that electron transport pathway in C II is FAD $\rightarrow$ [2Fe-2S] $\rightarrow$ [4Fe-4S] $\rightarrow$ [3Fe-4S] $\rightarrow$ Q. Haem b does not have to be involved in the direct electron transport, as edge to edge distance between [3Fe-4S] and Q is 7.1 Å

7

while between [3Fe-4S] and haem b 13.3 Å. Also redox potentials would favour direct transfer between [3Fe-4S] and Q. However, once reduced, electron may be passed from haem b to Q (Sun et al., 2005). Haem b was therefore proposed to act as an electron sink removing electrons from FAD and thus preventing electron leakage from flavin (Yankovskaya et al., 2003).
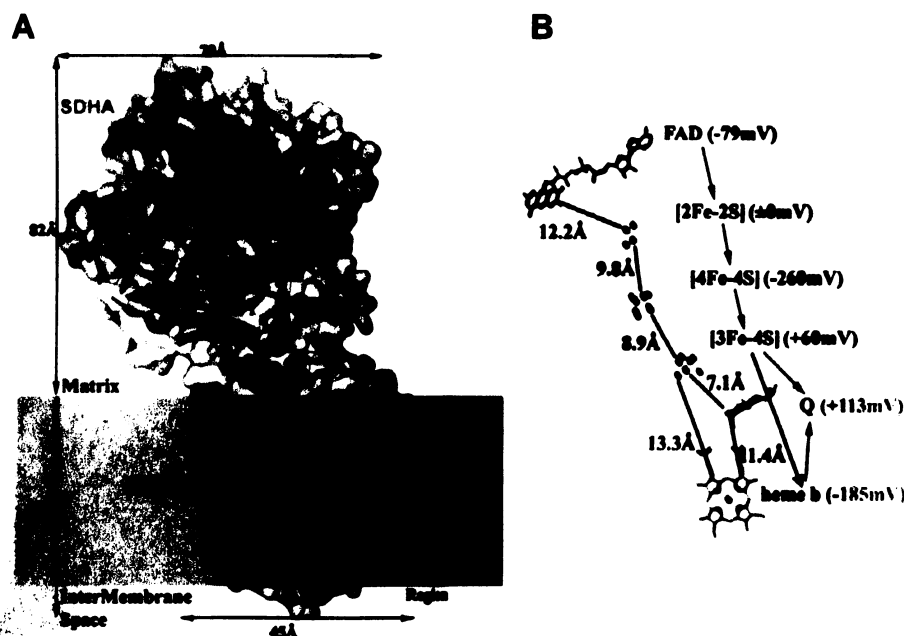
Figure 5: Crystal structure of bovine SDH. (A) Overall structure of the Complex II - the ribbon diagram of the complex is superimposed on the semitransparent molecular surface. FAD binding protein (SDHA) is shown in blue; iron-sulphur protein (SDHB) is shown in cream; the transmembrane subunits SDHC and SDHD are shown in pink and orange, respectively. The putative membrane region is shaded in grey. **(B)** The prosthetic groups constituting the electron transfer pathway (FAD, [2Fe-2S], [4Fe-4S], [3Fe-4S], and haem b) are shown together with ubiquinone (Q), along with their edge-to-edge distances and midpoint redox potentials. The electron transfer flow is indicated by arrows. Note that haem b does not lie in the direct transfer pathway. Adapted from (Sun et al., 2005).

Biochemical studies have also indicated that there are at least two ubiquinone binding sites in SDH – one site (Qp) is on the matrix side and the second (Qd) is near the intermembrane space side (Hagerhall, 1997). The two ubiquinone sites would result in branching of electron transfer. Ubiquinone at the Qp site would obtain electrons first, due to its favourable position. Then electrons would be transferred to ubiquinone in the Qd site after reduction at the Qp site becomes saturated. The presence of two ubiquinone binding sites favours the transfer of the two electrons from reduced FADH$_2$ to ubiquinones with

8

high efficiency which would further reduce possibility of electron leakage from flavin. Nevertheless, as a result semiquinones should be stabilized for a longer time to accept further electrons, which is in agreement with observed semiquinone signals in EPR studies (Ingledew et al., 1976; Miki et al., 1992). On the other hand, this would require good stabilization of semiquinone so as to prevent superoxide leak at this stage. Tyr-D91 at the Qp site is considered as the most important residue for the stability of the semiquinone (Yankovskaya et al., 2003). It is conserved in all SDH but is not found in QFR of *E. coli*, which is prone to electron leakage under aerobic conditions. Tyr-D61 at the Qd site is believed to play the same role in stabilizing semiquinone and is also conserved in mammalian mitochondrial SDH.

1.2.3 Mitochondrial glycerophosphate dehydrogenase

Mitochondrial glycerophosphate dehydrogenase is not included in the traditional scheme of respiratory chain, probably due to its very low expression in most tissues. However, it represents another dehydrogenase shuttling electrons to Q pool. It is involved in oxidation of cytosolic NADH by glycerophosphate shuttle which bypasses complex I (Bucher et al., 1958), in regulation of triglyceride synthesis (Kornacker et al., 1968) and possibly also in uncoupling protein-independent energy dissipation which involves also malic enzyme (Bobyleva et al., 1993; Bobyleva et al., 2000).

The content of mGPDH is highly tissue specific. The expression of the *mGPDH* gene is inducible by the thyroid hormone and glucocorticoids (Wilson et al., 1981). Transcription of *mGPDH* is activated by TR/RXR binding to the thyroid hormone response element (TRE) sequence in the promotor region of the gene (Weitzel et al., 2001; Yen et al., 1994).

The highest activity of mGPDH in equimolar proportion with cytosolic GPDH was found in BAT (Houstek et al., 1975). However, it is quite evident, that this enzyme plays an important role also in other tissues and cells. mGPDH was studied in pancreatic beta cells (Ishihara et al., 1996; Meglasson et al., 1989), brain (Cottingham et al., 1980a), heart muscle (Scholz et al., 1997), placenta (Swierczynski et al., 1976), testis (MacDonald et al., 1996), fibroblasts (Chretien et al., 1994) and after hormonal induction also in liver (Bobyleva et al., 2000). However, the multiple roles of mGPDH in cellular metabolism are not yet fully clarified and production of ROS could be another important feature of this enzyme.

9

Nuclear-encoded mGPDH is synthesized as an 81 kDa precursor which yields the 75 kDa mature protein containing one molecule of FAD (Garrib et al., 1986) (Figure 6). The enzyme is highly hydrophobic and membrane-assembled mGPDH forms oligomers (Garrib et al., 1986). mGPDH is extremely labile and solubilization and purification steps lead to gradual loss of enzyme activity (Beleznai et al., 1987; Cottingham et al., 1980a). Mammalian mGPDH exerts a high degree of homology with yeast and bacterial enzyme but no homology with the cytoplasmic NADH-linked cGPDH.

The enzyme shows complex allosteric kinetics which includes activating effect of calcium and other divalent cations (Wohlrab, 1977), inhibitory effect of free fatty acids (Houstek et al., 1975), acyl CoA esters (Bukowiecki et al., 1974) and glycolytic intermediates (Swierczynski et al., 1976) and also the dependence of enzyme activity on microviscosity of the mitochondrial membrane (Amler et al., 1990).
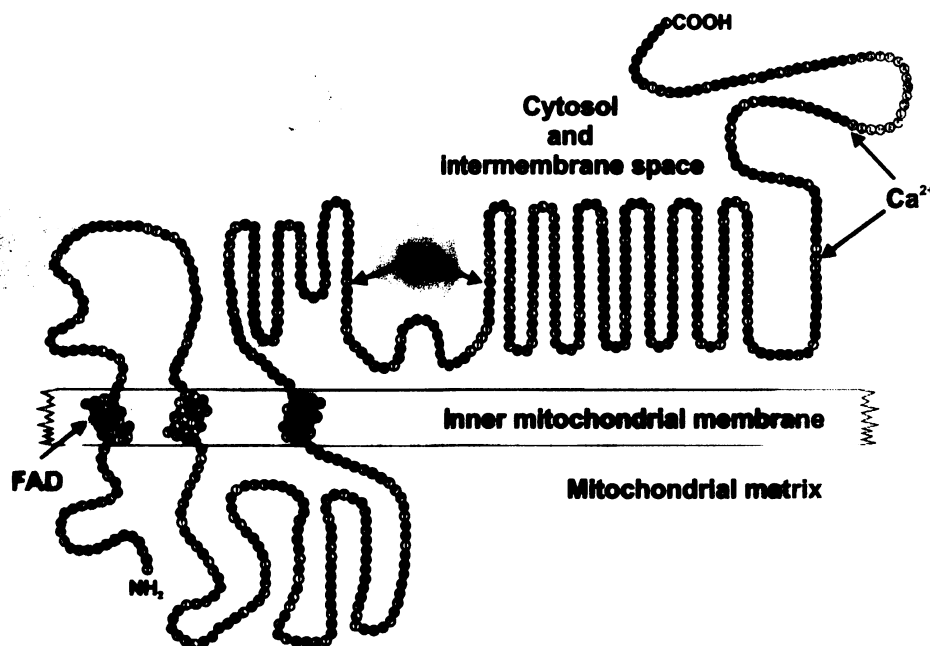
Figure 6: Schematic interpretation of proposed mGPDH topology. The mature protein has three transmembrane segments. N terminus is placed on the matrix side; the remaining 70% of the molecule lies in the intermembrane space, where both the calcium-binding and the GP-binding region are located. Adapted from (MacDonald et al., 1996).

To date there is no crystal structure of mGPDH, so the structure information is based only on predictions (Figure 6). A hydropathy plot (calculation of hydrophobic and

hydrophilic stretches in the protein sequence (Alberts, 2002)) suggested that the mature mGPDH protein has three transmembrane helices. These are centred at amino acids 80, 157 and 314, which places the FAD-binding domain into the middle of the first transmembrane segment (between residues 79 to 99). The N-terminus of the protein is localized in the matrix, as well as strongly positively charged loop between second and third transmembrane segment (amino acids 167 to 304). Almost 70% of the molecule at C-end is oriented towards intermembrane space. This part contains binding sites for glycerophosphate (amino acids 393-405 and 428-445) as well as for calcium (amino acids 636-647) (MacDonald et al., 1996). Active site is therefore accessible from cytosolic side. This is in agreement with the finding, that glycerophosphate does not have to traverse the inner mitochondrial membrane to be oxidized (Klingenberg, 1970). Location of the calcium binding domain, which shares homology with E-F hand sequence in calmodulin, offers the possibility for regulation of mGPDH activity by cytosolar calcium.

1.2.4 Complex III – ubiquinol: cytochrome c reductase

The complex III transfers electrons from ubiquinone to cytochrome c. The complex III comprises of 11 subunits. It is functional as a dimer, with the two monomers acting in dependence on each other. Molecular weight of the dimer is approximately 480 kDa (Capaldi, 1982). Only one of the subunits - cytochrome b - is mitochondrially encoded. The redox groups in cyt bc_1 comprise a 2Fe/2S centre located on Rieske protein; two b type haems (high potential haem b_H aka b_{562} and low potential b_L aka b_{566}) on a single polypeptide and the haem of cyt $c1$. Both Rieske protein and cytochrome $c1$ comprise of globular domain incorporating the redox centre and hydrophobic anchor. The cyt b subunit has eight transmembrane α-helices; four conserved histidine residues on them position the haems: b_L towards the cytoplasmic and b_H towards the matrix side of the mitochondrial membrane. Only the three subunits containing redox centres play role in reduction of cytochrome. The other eight subunits do not have catalytic role. One of the small subunits targets another of the subunits to the mitochondrion from its site of synthesis in cytoplasm, while two of the others are peptidases that may catalyze removal of targeting sequences (Braun et al., 1995).

11

1.2.4.1 The Q cycle

The mechanism of electron transfer/proton translocation coupling is known as the Q cycle (Mitchell, 1975). As it is crucial for the mechanism of ROS production on complex III, it should be described here in more detail (Figure 7).
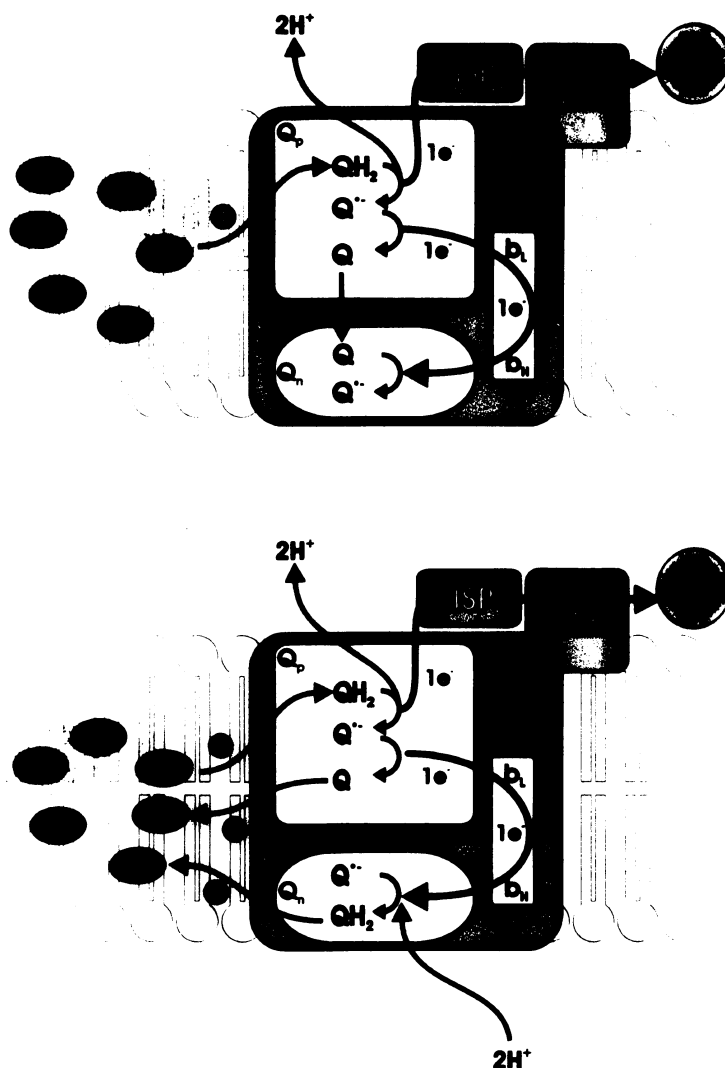
Figure 7: Scheme of the Q cycle. Two QH_2 molecules are subsequently taken into the cycle, one is returned back to Q pool at the end. At Q_p, QH_2 undergoes two step oxidation, first electron is transferred via Rieske protein (ISP) to cyt c, second reduces $Q^{\cdot-}$ at Q_n. Red arrows depict transfer of electrons, ❶ and ❷ stands for first and second coenzyme Q molecule taken into the cycle. Adapted from (Nicholls et al., 2000).

Stage 1 – QH_2 oxidation at Q_p. The pool of ubiquinone and ubiquinol in inner mitochondrial membrane is in a large excess over other components of the respiratory chain. A molecule of QH_2 from this pool diffuses to a binding site Q_p, close to the

cytoplasmic side of the membrane and adjacent to Rieske protein. The oxidation of QH_2 then takes place in two steps – the first electron is transferred from QH_2 to Rieske protein, releasing two protons to the cytoplasm and leaving semiquinone ($Q^{\bullet -}$) at the Q_p site. In the next step, the second electron is transferred to the b_L haem, which is also close to cytoplasmic side. The $E_{m,7}$ for the $QH_2/Q^{\bullet -}$ couple is +280mV, thus being 220mV more positive than $E_{m,7}$ of two electron oxidation (+60mV). This implies that the second step of the oxidation will be energetically favourable, as the $Q^{\bullet -}$ seeks to lose its second electron. Under some circumstances this might be transferred directly to molecular oxygen, thus generating superoxide anion.

Stage 2 – Q reduction to $Q^{\bullet -}$ at Q_n. The electron from haem b_L ($E_{m,7}$ -100mV) passes to the haem b_H ($E_{m,7}$ +50mV). Although the difference between redox states seems to be +150mV, in practice, due to positioning of b_L and b_H haems on opposite sides of the membrane, it is counteracted by $\Delta\Psi_m$ ($\approx$ 150mV), and therefore close to equilibrium. In uncoupled mitochondria, e^- does not pass against the electrochemical gradient and energy is therefore dissipated at this step. On the other hand, very high values of $\Delta\Psi_m$ will retard electron transfer between b type haems, leading to the prolongation of Q_p site occupancy by semiquinone and enhancing the chances of $O_2^{\bullet -}$ production. From b_H haem e^- is passed to Q bind at Q_n site which is located in the close vicinity of b_H and subsequently $Q^{\bullet -}$ is formed.

Stage 3 – $Q^{\bullet -}$ reduction to QH_2 at Q_n. In the last part, second molecule of QH_2 is oxidized at Q_p in a repetition of stage 1 with one electron passing to cyt $c1$ and the other via b_L to b_H. This second electron reduces $Q^{\bullet -}$ bound to Q_n to QH_2; QH_2 then returns to the bulk pool and the cycle is completed.

In summary, total amount of 4 protons is translocated, one QH_2 molecule is oxidized to UQ and two cyt c molecules are reduced throughout the cycle.

1.2.5 Complex IV – cytochrome *c* oxidase (COX)

Terminal site of the respiratory chain - complex IV transfers electrons to O_2. The molecular weight of mammalian cytochrome c oxidase is 205 kDa. The COX is integral membrane complex, in majority of higher eukaryotes composed of 13 subunits. Catalytic core of the enzyme is formed by the three largest subunits: COX1, COX2 and COX3. These three subunits are encoded by mtDNA, while nuclear genome encodes the ten remaining "small" subunits.

13

The enzyme contains four redox centres: two haem centres haem a_3 and haem a both belonging to identical group of A haems and two Copper ions CuA and CuB (Froncisz et al., 1979; van Gelder et al., 1969). The haem a_3 and CuB are close to each other and form binuclear centre, where reduction of oxygen to water occurs.

The small nuclear subunits play a regulatory role. Enzyme activity may be influenced by their phosphorylation, binding of ATP, ADP and other allosteric factors. In mammals are some of these subunits (COX4, COX6a, COX7a, COX8) expressed as tissue specific isoforms, which offers further regulatory properties.

In case of COX, proton translocation is not directly linked to reduction of electron carrier. The electrons first flow from cytochrome c to CuA centre, then they are transferred to cytochrome a in subunit COX1 and further on to bimetallic CuB/haem $a3$ active site. The active site is connected with mitochondrial matrix by two hydrophilic channels D and K – protons are translocated via these channels during oxygen reduction.

1.2.6 Complex V – F_1F_O - ATP synthase

The mitochondrial ATP synthase (ATPase) utilizes membrane potential of protons formed by RC complexes to synthesize ATP. The enzyme complex is complicated molecular machine, which is capable of either ATP synthesis or proton pumping at the expense of ATP hydrolysis. The ATPase enzyme complex has approximate molecular weight of 670 kDa. It is composed of a catalytic F_1 part connected by two stalks with a membrane-embedded F_0 part. The mammalian enzyme is built of at least 16 different subunits (F_1: $\alpha_3\beta_3\gamma\delta\epsilon$ + IF1, F_0: a, b, c_{10-14}, d, e, f, g, F_6, A6L, OSCP, (factor b)) (Belogrudov, 2002; Collinson et al., 1994) of which two F_0 subunits - the subunit a (subunit 6) and subunit A6L are encoded by mtDNA.

Biosynthesis of eukaryotic ATPase is a highly ordered process, which involves several ATPase-specific assembly proteins. Studies in yeast identified five chaperone-type factors necessary for the assembly of the functional enzyme. Atp10p and Atp22p were found to mediate F_0 assembly (Ackerman et al., 1990; Helfenbein et al., 2003) while Atp11p, Atp12p and Fmc1p are required for the F_1 part (Ackerman et al., 1990; Lefebvre-Legendre et al., 2001). In mammalian cells only the orthologues of yeast *ATP11* and *ATP12* but no F_0–specific assembly factors have been found (Pickova et al., 2005). Like the yeast proteins, human Atp11p has chaperone-like activity toward the β subunit and human Atp12p interacts with the α subunit (Hinton et al., 2004; Wang et al., 2001).

F_oF_1 ATPase represents a unique motor protein, composed of two opposing rotary motors connected in series: the transmembrane F_o motor is fuelled by the ion-motive force, while the soluble F_1 motor is fuelled by nucleotide hydrolysis. The flow of protons through the proton channel generates conformational energy in subunits a and A6L, which is transformed into the rotation of c subunits ring. This drives the movement of γ shaft which relays this movement to the F_1 sector and induces cyclic conformational changes of the adenine nucleotide binding pockets (Oster et al., 2003).

1.3 Mitochondrial ROS production

The standard reduction potential for the conversion of molecular oxygen to O_2^- is - 0.160 V. And in the respiratory chain there is a variety of redox centres with standard reduction potentials between -0.32 V (NAD(P)H) and +0.39 V (cytochrome $a3$ in complex IV). Given the highly reducing intramitochondrial environment, various respiratory components, including flavoproteins, iron–sulfur clusters and ubisemiquinone, are thermodynamically capable of transferring one electron to oxygen. Moreover, most steps in the respiratory chain involve single-electron reactions, further favouring the monovalent reduction of oxygen. Also the fact that superoxide is readily removed from the reaction by numerous ROS detoxifying systems makes superoxide production practically irreversible reaction (Turrens, 2003).

Over the past 30 years variety of mitochondrial sources of O_2^- have been identified. Currently, eleven different mitochondrial enzymes are considered to be potential sources of ROS (see Table 1 for summary). Superoxide formation occurs on the outer mitochondrial membrane, in the matrix and on both sides of the inner mitochondrial membrane. Whilst the O_2^- generated in the matrix is eliminated in that compartment, part of the O_2^- produced in the intermembrane space may be carried to cytoplasm via voltage-dependent anion channels (Han et al., 2003). Table 1 and Figure 8 give the overview of ROS producing sites in mitochondria. The following chapters then discuss in more detail molecular aspects of ROS production by respective enzyme complexes.

Table 1: Sites and topology of mitochondrial ROS production (adapted from (Turrens, 2003) and updated)

Component	Localisation / Topology	Primary ROS produced	References
Complex I (NADH dehydrogenase)	Inner membrane/ inner side	$O_2^{-\bullet}$	(Turrens et al., 1980)
Complex II (succinate dehydrogenase)	Inner membrane/ inner side	$O_2^{-\bullet}$	(Lenaz, 2001; Zhang et al., 1998a)
Complex III (ubiquinol-cytochrome c reductase)	Inner membrane/ inner side	$O_2^{-\bullet}$	(Boveris et al., 1976; Cadenas et al., 1977)
	Inner membrane/ outer side (both sides)		(Han et al., 2001; Miwa et al., 2005)
Complex IV (cytochrome c oxidase)	Inner membrane/ inner side	$O_2^{-\bullet}$, NO	(Prabu et al., 2006) (Castello et al., 2006)
Mitochondrial glycero-phosphate dehydrogenase	Inner membrane/ outer side	$O_2^{-\bullet}$	(Drahota et al., 2002)
	Inner membrane/ both sides		(Miwa et al., 2005)
External NADH dehydrogenase	Inner membrane/ outer side	$O_2^{-\bullet}$	(Fang et al., 2003)
Dihydroorotate dehydrogenase	Inner membrane/ outer side	H_2O_2	(Forman et al., 1976)
Monoamine oxidase	Outer membrane/ inner side	H_2O_2	(Cadenas et al., 2000)
Cytochrome $b5$ reductase	Outer membrane	$O_2^{-\bullet}$	(Whatley et al., 1998)
α-ketoglutarate dehydrogenase	Matrix	$O_2^{-\bullet}$	(Starkov et al., 2004)
Aconitase	Matrix	OH•	(Vasquez-Vivar et al., 2000)

1.3.1 ROS production by complex I

The mechanism of superoxide production by complex I has not been elucidated yet, probably because the exact electron transport sequence through CI as well as the

mechanism of coupling are not known (Degli Esposti et al., 1994). However, the recent crystallization of bacterial complex I and description of electron transfer pathway in this case (Sazanov et al., 2006) have been bringing signs that in the near future the molecular mechanism of C I ROS production will be solved.

Most of the redox centres in C I were suggested so far as the ROS producing sites. On thermodynamic grounds, centre N1a (Kushnareva et al., 2002) and the flavin (Kudin et al., 2004) were suggested as the main superoxide producing sites. Based on inhibitor studies, it was again the flavin (Liu et al., 2002), centre N2 (Genova et al., 2001) and the iron-sulphur proteins and semiquinones in general (Herrero et al., 2000). It is feasible that all these sites produce superoxide and that production rates by different sites are tissue or condition specific.

An interesting observation reported in several studies is that mitochondria respiring on succinate, the substrate for complex II (in the absence of rotenone, an inhibitor of complex I), have a greater rate of superoxide production than they do when respiring on complex I-linked substrates (Kushnareva et al., 2002; Liu et al., 2002; Turrens et al., 1980). It was hypothesized that most of the succinate driven superoxide production occurs during reverse electron transport into complex I (Figure 9) (Han et al., 2003; Liu et al., 2002; Votyakova et al., 2001), and thus superoxide production during reverse electron transport is greater than during forward electron transport. The mechanism and physiological relevance of this phenomenon are not known. Over the last years, it has become apparent that the rate of superoxide production by the electron transport chain *in vitro* is sensitive to the mitochondrial protonmotive force (Δp) (Korshunov et al., 1997; Votyakova et al., 2001). This conclusion is based on observations that addition of either uncouplers (which dissipate Δp) or inhibitors (which inhibit formation of Δp) decreases the rate of superoxide production by mitochondria respiring on succinate in the absence of rotenone. Reverse electron transport depends on the thermodynamic forces across complex I and is, therefore, favoured by a high Δp and a high reduction state of the Q pool. However, in the intact electron transport chain, Δp will have both a direct effect on complex I and an indirect effect through the Q pool, because of its downstream effects on complex III and complex IV. Lowering Δp will tend to oxidize the Q pool and decrease reverse electron supply into complex I, and indirectly lower superoxide production (Lambert et al., 2004b). These complications make it difficult to assess the relative importance of the direct and indirect effects of Δp on superoxide production by complex I.

1.3.2 ROS production by complex III

Already in 1970's mitochondrial complex III was proposed as a massive source of superoxide (Boveris et al., 1976) and Rich and Bonner were first to recognize, that an unstable semiquinone formed at Q_p site is responsible for superoxide formation (Rich et al., 1978). The mechanism of superoxide production by complex III is relatively well understood, as has already been mentioned in chapter 1.2.4.1 When electron flow through complex III is retarded by high membrane potential or blocked by inhibitor of Q_n site (antimycin A) $Q^{•-}$ persists for long time in Q_p site. $Q^{•-}$ is a strong reductant and as Q_p site is accessible for oxygen, an electron might escape from semiquinone to oxygen, thus forming $O_2^{•-}$. This effect is suppressed by Q_p inhibitors such as myxothiazol and stigmatellin (Raha et al., 2000; Turrens et al., 1985). Myxothiazol can also induce superoxide production at Q_p site when the quinone pool is highly reduced, albeit at smaller rates than antimycin A (Raha et al., 2000; Starkov et al., 2001).

Q_p site is located in the hydrophobic domain on the cytosolic side of the membrane, which led to an early predictions that complex III produces superoxide towards intermembrane space (Zhang et al., 1998b). Quite surprisingly, two recent reports that studied topology of superoxide production proposed that 50 to 70% of superoxide is produced towards matrix side (Miwa et al., 2005; Muller et al., 2004).

Given that the reactions between semiquinone and oxygen at complex III may occur within the membrane bilayer, it is not intuitive, how can superoxide reach both the inner and the outer surface of the membrane. Some mechanistic speculations were proposed concerning movement of superoxide within membrane. According to these speculations neutral $HO_2^{•}$ may be formed at Q_p and then dissociate to superoxide (and H^+) when it contacts the aqueous phase or directly the neutral semiquinone may escape from Q_p, reach either side of the membrane and only here react with oxygen to form superoxide (Muller et al., 2002). By analogy, these explanations could also be applied to glycerol phosphate dehydrogenase if the reaction site(s) are in the hydrophobic phase of the membrane.
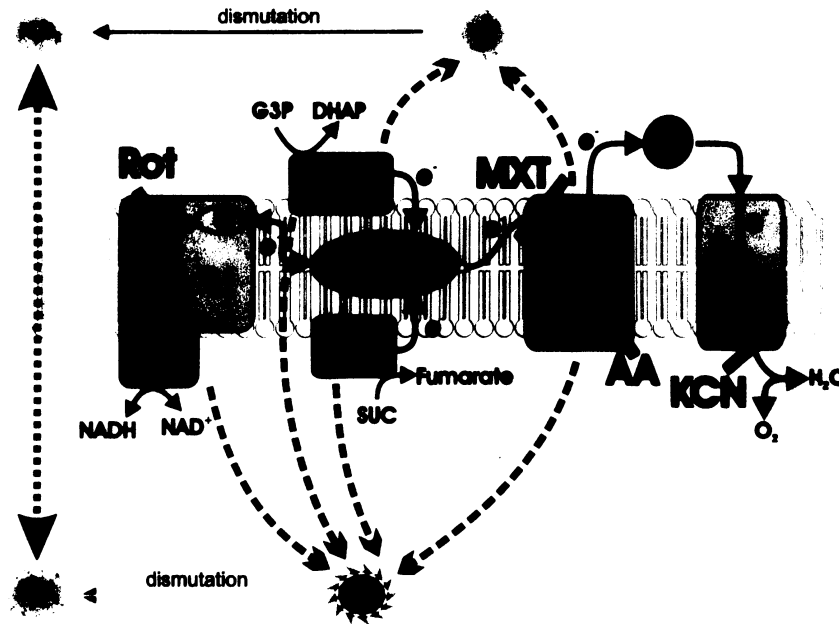
Figure 8: Sites and topology of ROS production in mitochondrial respiratory chain. Electron flow (black arrows), sites of ROS production (blue arrows) as well as sites of action of individual OXPHOS inhibitors (red dashes) are depicted. The topology of ROS production is indicated by the rientation of blue arrows. Note that some complexes produce ROS to both sides of the membrane. Abbreviations: mGPDH – mitochondrial glycerophosphate dehydrogenase; Q – coenzyme Q; C I, C II, C III and C IV - complexes of respiratory chain; Rot – rotenone MXT – myxothiazol; AA – antimycin A; KCN – cyanide.

1.3.3 ROS production by mGPDH

Data in literature and our studies indicate that mechanism of GP-dependent ROS generation proceeds by different pathways than that connected with complex I and complex III function. Recent studies in *Drosophila* mitochondria show that ROS generation by mGPDH depends to much lower extent on mitochondrial membrane potential $\Delta\Psi_m$, and contrary to complex I the ROS produced by mGPDH are generated towards the intermembrane space (Miwa et al., 2003).

Our studies further indicate that the intensity of mGPDH-dependent ROS production relative to specific content of the enzyme could be higher than that connected with complex I, III and KGDH. Therefore its detrimental effect on cellular metabolism could be much more significant (Drahota et al., 2002).

Hydrogen peroxide may be formed in analogy with mechanism proposed for ROS production by complex I and III from ubisemiquinone radical III (Barja et al., 1998; Kwong et al., 1998; Turrens et al., 1986). Our data, however, indicate that there exists

difference in the transport of reducing equivalents from mGPDH and succinate dehydrogenase to the Q pool. GP-induced ROS production is higher than that induced by succinate. This difference could be due to the fact that whereas a specific Q-binding protein was detected in succinate dehydrogenase (Yu et al., 1978), it appears to be missing in mGPDH (Cottingham et al., 1980a; Cottingham et al., 1980b; Rauchova et al., 1992). This Q-binding protein evidently represents a natural protection of ubisemiquinone formed during Q reduction by succinate dehydrogenase. Only in situations when reduced flavoprotein in succinate dehydrogenase has no accessible electron transfer partner, higher portion of ubisemiquinone may react under specific conditions with oxygen (Zhang et al., 1998a). Due to the absence of Q-binding protein ubisemiquinone formed by mGPDH may be more accessible to oxygen.

Absence of the protective effect of Q-binding protein thus could explain both the higher ROS production induced by GP than by succinate in the presence of antimycine A. However we can not exclude, that the hydrogen peroxide is formed directly by the interaction of molecular oxygen with reduced flavoprotein moiety of the mGPDH similarly as proposed for the acetyl CoA dehydrogenase (Osumi et al., 1978).

1.3.4 Other sites of mitochondrial ROS production

This chapter completes the list of mitochondrial ROS producers. Support for some of them, such as cytochrome $b5$ reductase is fairly weak, while others (monoamine oxidase) are by no means important sources of ROS, but their activity is not directly connected to mitochondrial energetic metabolism and therefore are not dealt with here in more detail.

Succinate dehydrogenase – As mentioned already in chapter 1.2.2, SDH is well protected from electron leak and under physiological conditions it does not seem to be ROS producer. However, there are studies on isolated enzyme, either bacterial (Messner et al., 2002) or bovine (Zhang et al., 1998a), which demonstrate that reduced FAD of succinate dehydrogenase might donate electron to oxygen and produce O_2^- in the absence other immediate electron acceptor (coenzyme Q). Also under pathophysiologic conditions, when the enzyme is mutated, it may become significant ROS producer. This is supported by several lines of evidence. First, mutation in SDHC of *C. elegans* results in a dramatic decrease of life span, accompanied by accumulation of protein carbonyls and increase O_2^- in production (Ishii et al., 1998; Senoo-Matsuda et al., 2003). Also some mutations in

human SDHD can result in situations promoting ROS production. For example mutation HisD79 → Leu destroys the ligation of haem b and lowers its redox potential by 100 mV. This might potentially contribute to distribution of electrons towards FAD and O_2^{-} production (Baysal et al., 2000; Sun et al., 2005).

Cytochrome *c* oxidase – Although there is a general agreement that cytochrome *c* oxidase is well protected against electron leak and may not be a source or ROS, there is one recent report describing ROS production by liposome reconstituted COX with ferrocytochrome *c* as a substrate (Prabu et al., 2006). However, liposomes in their experiments do not seem to be free of other OXPHOS complexes and even methodological issues could be raised. But accidentally, there is another recent report claiming, that under hypoxic conditions COX switches its function from O_2 reductase to NO_2^{-} reductase and produces NO instead of H_2O (Castello et al., 2006). This may interfere with ROS measurements in previous case and produce false positive results. As well, this finding may contribute to explaining paradoxical increase in ROS production under hypoxic conditions.

α-ketoglutarate dehydrogenase – is an enzyme complex associated with inner side of the inner mitochondrial membrane. This enzyme is a part of tricarboxylic acid cycle, where it catalyzes oxidation of α-ketoglutarate to succinyl-CoA. It is composed of multiple copies of three subunits (E1k, E2k and E3). The enzyme has recently been proposed as a source of superoxide and hydrogen peroxide. E3 subunit (dihydrolipoamide dehydrogenase, identical with E3 subunit of pyruvate dehydrogenase) seems to be responsible for ROS production. ROS production is stimulated by low availability of NAD^{+}, natural electron acceptor of KGDH. States where mitochondrial NAD^{+}/NADH pool is greatly reduced (CI inhibition, high $\Delta\Psi_m$) should therefore promote KGDH-dependent ROS production (Starkov et al., 2004).

Aconitase – is another enzyme of tricarboxylic acid cycle localized in mitochondrial matrix. It catalyzes conversion of citrate to isocitrate. Its iron-sulphur cluster is very easily inactivated by superoxide and thus aconitase activity often serves as a marker of oxidative stress. It was also demonstrated that upon inactivation, aconitase induces production of highly dangerous hydroxyl radical. Fe^{2+} released from iron-sulphur cluster is most likely responsible for this hydroxyl production (Vasquez-Vivar et al., 2000).

21

Cytochrome *b*5 reductase – is integral enzyme of outer mitochondrial membrane. It oxidizes NAD(P)H and reduces cytochrome *b*5 of the outer membrane. It may also reduce ascorbyl free radical and thus regenerate ascorbate in liver. It was shown to be capable of high rate superoxide production (Whatley et al., 1998).

Monoamine oxidase – is an enzyme located in outer mitochondrial membrane. It catalyzes oxidation of biological amines – reaction accompanied with H_2O_2 production. In brain mitochondria it plays a central role in the turnover of monoamine neurotransmitters. Intensity of ROS production by MAO might be very high; in fact it can outperform the whole ROS production by the respiratory chain. It was proposed to be the main source of H_2O_2 in ischemic tissues. Some authors also suggest that upregulation of MAO resulting in increased H_2O_2 production is one of the pathogenic mechanisms in Parkinson's disease (Cadenas et al., 2000).

Dihydroorotate dehydrogenase – is an enzyme from the outer surface of the inner mitochondrial membrane ubiquitously expressed in mammalian tissues. It is involved in synthesis of pyrimidine nucleotides, where it catalyzes conversion of dihydroorotate to orotate. *In vitro* it produces H_2O_2 in the absence of coenzyme Q – its natural electron acceptor (Forman et al., 1976). The significance of this process *in vivo* is questionable.

External NADH dehydrogenase – besides complex I several other NADH dehydrogenases exists in plants, yeast and fungi. The two external NADH dehydrogenases in *S. cerevisiae* function in the re-oxidation of the cytosolic NADH produced by glycolysis. To date, there is one report that suggests these enzymes as a potential source of superoxide (Fang et al., 2003).

1.3.5 Relative contribution of individual ROS sources

It is very hard to make any estimates on relative involvement of individual mitochondrial complexes in overall ROS production, because most of the available data comes from isolated mitochondria (or even worse from submitochondrial particles) subjected to non-physiological conditions. Nevertheless, when trying to extract some knowledge from available data it is evident that: (1) the relative contribution of every site

to the overall O_2^- production shows high tissue dependency and (2) redox state of the mitochondria is very important (Barja, 1999).

Based on the mitochondria data it seems that complex III is responsible for most of the O_2^- production in heart and lung mitochondria (Turrens et al., 1980; Turrens et al., 1982) while complex I appears to be the primary source of O_2^- in the brain (Barja, 1999; Barja et al., 1998). The contribution of complex III in overall ROS signal may significantly depend on the concentration of coenzyme Q in mitochondrial membrane of respective tissue. There is a strong correlation between rates of H_2O_2 release and ubiquinone content in mitochondria of different sources (Turrens et al., 1982).
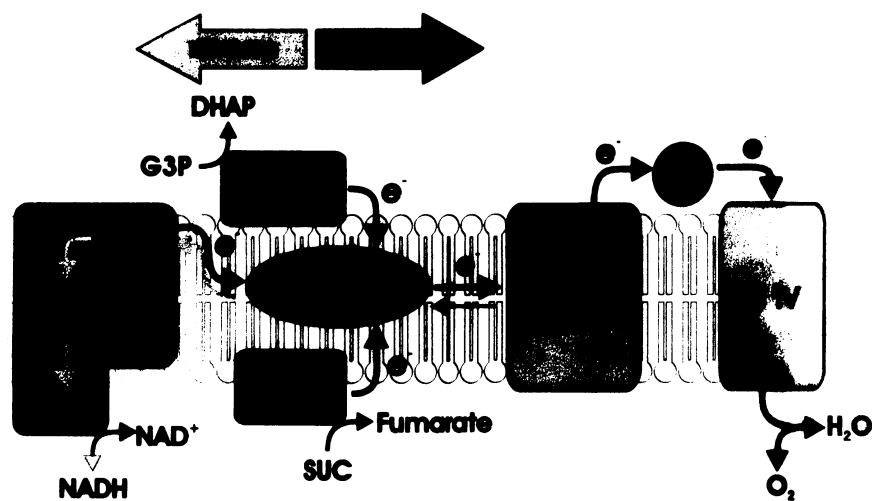

Figure 9: Forward and reverse electron transport in the respiratory chain. Dehydrogenases supplying electrons to Q pool are in blue, components downstream of Q pool are in green. The forward flow of electrons through OXPHOS is depicted by red arrows, while the reverse flow is indicated by yellow arrows.

Another important issue worth considering is the relationship between ROS production and $\Delta\Psi_m$. It is documented that high levels of $\Delta\Psi_m$ above 140 mV lead to an exponential increase of mitochondrial ROS production (Korshunov et al., 1997, Votyakova 2001; Liu, 1999). In line with this finding, decrease of $\Delta\Psi_m$ via stimulation of ATP synthase activity, a low ATP/ADP ratio, substrate limitation or increased proton permeability lower the amount of ROS produced (Kadenbach, 2003). Most, if not all, of this $\Delta\Psi_m$-sensitive ROS production can be ascribed to the phenomenon of reverse electron flow from succinate to NAD. Under these conditions complex I produces superoxide at very high rates (Kushnareva et al., 2002; Liu et al., 2002; Turrens et al., 1980; Votyakova

et al., 2001) in isolated mitochondria. Unfortunately the physiological relevance of reverse electron transport still remains unclear.

The original studies identified $\Delta\Psi_m$ to be responsible for regulation of ROS production. But recently the relative contribution of the two components of protonmotive force ($\Delta\Psi_m$ and ΔpH) was studied. Surprisingly, ΔpH component turned out to be the one required for high rates of superoxide production by complex I during reverse electron transport (Lambert et al., 2004a; Lambert et al., 2004b).

What does this mean from the physiological point of view? First, as mentioned above, there is still not enough support for existence of reverse electron flow under physiological conditions. We can only make assumptions based on mitochondrial experiments. Secondly, if $\Delta\Psi_m$ would be the key regulator, we could assume that staying at state 4 with high membrane potential and all components of respiratory chain reduced is the most dangerous condition for the cell. But thirdly if ΔpH is to be blamed, we need another explanation. As was mentioned in chapter 1.1.3 Δp consists mostly from $\Delta\Psi_m$ under normal conditions. Redistribution from $\Delta\Psi_m$ to ΔpH occurs if an electrically permeant ion is transported to mitochondria. Physiologically this occurs for example during uptake of Ca^{2+}. Calcium overload might thus be the most dangerous condition leading to a boost of ROS production. However, to what extent are these hypotheses plausible, it still remains to be proved in the future. Furthermore, highly reduced respiratory chain seems not only to promote ROS production directly from complex I but also indirectly, via $NADH/NAD^+$ pool, from KGDH (Starkov et al., 2004).

If these processes occur physiologically, one may expect that they may also be regulated at different levels. First of all, UCP proteins were proposed as important regulators of oxidative stress. It was shown that an increase in superoxide concentration caused activation of UCPs resulting in mild uncoupling (Echtay et al., 2002a; Echtay et al., 2002b). It indicated that the interaction of superoxide with UCPs might be a mechanism for decreasing Δp and thus preventing the ROS production inside mitochondria.

An additional recent proposal for ROS regulation is that the entry of electrons into and through the OXPHOS, especially at the level of complex I, is highly regulated. This electron gate would presumably only permit oxidative phosphorylation to occur when it was required by cellular energetic needs (Bose et al., 2003; Joubert et al., 2004). Such mechanism potentially permits the modulation of oxidant formation without the requirement to dissipate Δp through the energetically non-productive action of UCPs. There is also a growing appreciation that the activity of OXPHOS enzymes can also be

24

altered by covalent modification and that these modifications may be important in ROS formation (Ludwig et al., 2001).

1.4 Mitochondrial antioxidant defence mechanisms

The deleterious effects of ROS produced in mitochondria have to be overcome by various antioxidant systems. These systems are designed to eliminate both O_2^- and H_2O_2 and are very effective. As a result, the steady state concentrations of O_2^- and H_2O_2 have been estimated to be as low as 10^{-10} M and 5×10^{-9} M, respectively (Cadenas et al., 2000).

Superoxide is the most common radical produced in mitochondria. Since O_2^- may either reduce transition metals, which in turn can react with H_2O_2 producing OH• or spontaneously react with NO• to produce peroxynitrite, it is important to maintain steady state concentrations of O_2^- as low as possible. This is achieved by a family of metalloenzymes called superoxide dismutases (SOD), which enzymatically convert O_2^- to H_2O_2. Although the dismutation of O_2^- to H_2O_2 can also occur spontaneously, the role of SOD is to increase rate of this reaction to that of a diffusion controlled process.

Since O_2^- can hardly pass through the membranes, independent mechanisms exist for its detoxification in matrix and in intermembrane space (Figure 10). The mitochondrial matrix contains a specific form of SOD with manganese in the active site – MnSOD (SOD2). Expression of MnSOD is induced by agents that cause oxidative stress via oxidative activation of NFκB. The steady state concentration of O_2^- in the intermembrane space is controlled by three different mechanisms. Firstly, this compartment contains a different isozyme of SOD, with copper and zinc instead of manganese – CuZnSOD (SOD1), which is also found in cytoplasm of eukaryotic cells. Secondly, the intermembrane space contains also high concentration of cytochrome c, which can react with O_2^- (k = 10^7 $M^{-1}s^{-1}$), regenerating oxygen in this process. The reduced cytochrome c can then transfer electrons to COX. Thus, some of the electrons that escaped the respiratory chain producing O_2^- may re-reduce cytochrome c and still contribute to energy production. Finally, lower pH in the intermembrane space facilitates spontaneous dismutation of O_2^- (Turrens, 2003).
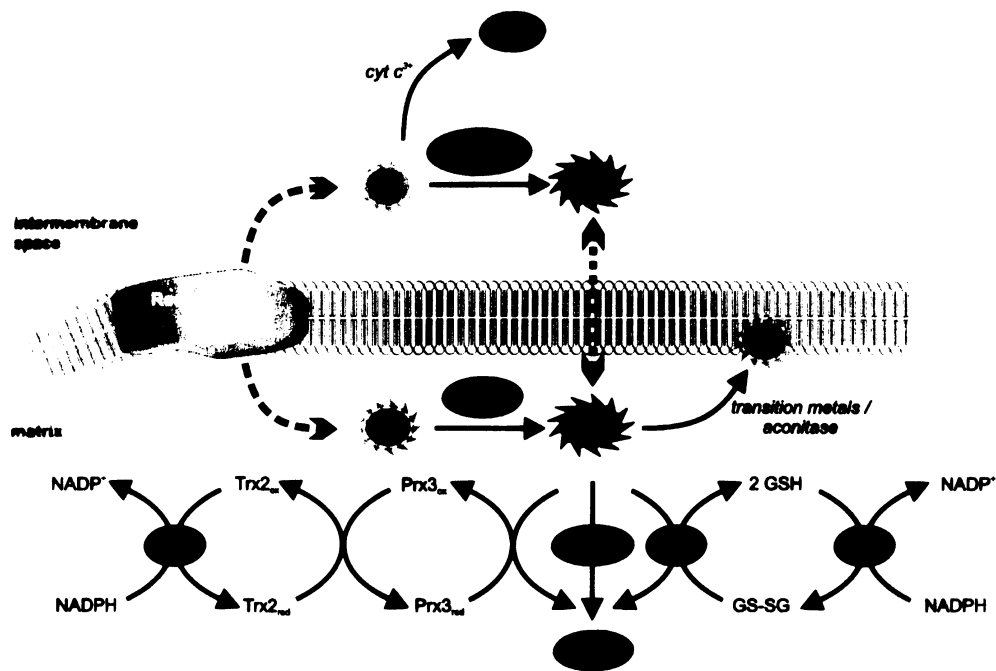
Figure 10: Cellular antioxidant defenses. Mechanisms involved in detoxification of O_2^- and H_2O_2 are shown. Note that while O_2^- can not cross the membrane H_2O_2 may diffuse freely. Cu/Zn SOD - cytosolic superoxide dismutase, MnSOD - mitochondrial superoxide dismutase, cyt c - cytochrome c, GPx – glutathione peroxidase, GR – glutathione reductase, Prx – peroxiredoxin, Trx – thioredoxin, TrxR$_2$ – thioredoxin reductase.

H_2O_2, the product of O_2^- dismutation is decomposed by several ways (Figure 10). Probably the most important is represented by glutathione peroxidase (GPx). In liver, mitochondria account for about one third of the total GPx activity. A second glutathione peroxidase associated with the mitochondrial membrane, known as phospholipid-hydroperoxide glutathione peroxidase, is specifically involved in reducing lipid peroxides associated with the membrane.

H_2O_2 may also be detoxified by peroxiredoxins – the recently discovered family of thioredoxin dependent peroxide reductases (Fujii et al., 2002; Wood et al., 2003a; Wood et al., 2003b). So far there are two mitochondrial isoforms: Prx3 and Prx5. Both Prx3 and Prx5 are regenerated in their active form by disulfide oxidoreductase thioredoxin (Trx2), which in turn is reduced by thioredoxin reductase (TrxR2). As TrxR2 utilizes NADPH as a source of reducing equivalents, this system is dependent on functioning oxidative phosphorylation in mitochondria. Apart from ROS detoxification, thioredoxins play multitude of other cellular functions. This system might be essential for normal mammalian development, as knock-out of Trx2 is embryonally lethal (Nonn et al., 2003).

26

Catalase, a major H_2O_2 detoxifying enzyme found in peroxisomes, is also present in heart mitochondria. However, this enzyme has not been found in mitochondria from other tissues, including skeletal muscle.

In addition to cytochrome c, other electron carriers appear to have detoxifying role against ROS. Ubiquinol has been shown to act as a reducing agent in the elimination of various peroxides in the presence of succinate. Thus, coenzyme Q may act both as a source of ROS (partially reduced semiquinone) and as an antioxidant (fully reduced ubiquinol). The inner mitochondrial membrane also contains vitamin E, another powerful antioxidant that acts in the lipid phase and interferes with free radical-mediated chain reactions.

1.5 Measuring of cellular ROS production

Measuring of ROS production is prone to many artifacts and very conflicting results are published at the moment. Basically the methods can be divided into (1) trapping of ROS or (2) measuring of the damage caused by ROS. Any trapping, either by spin-traps for EPR or by reaction of ROS with fluorescent probes, effectively scavenges ROS from the system and therefore shifts the balance present in cell. This may in turn promote ROS production and thus lead to its overestimation in cells. But neither the subsequent detection of the damage is trouble free. It is always very difficult to ascribe some parameter (DNA damage, protein carbonylation, lipid oxidation) specifically to mitochondrial ROS and it is even harder to make any quantitative estimates.

More details may be found in extensive reviews elsewhere (Halliwell et al., 2004). Here I summarize only potential pitfalls connected to measuring with probes we used in our experiments.

1.5.1 Dichlorodihydrofluorescein

Dichlorodihydrofluorescein (H_2DCFDA) and its derivatives (Carboxy-H_2DCFDA, CM-H_2DCFDA) are widely used as probes for measurement of redox activity in intact cells (Degli Esposti, 2002). DCFDA is oxidized by hydroxyl, peroxyl and alkoxyl radical and peroxynitrite, while neither H_2O_2 nor $O_2^{-\cdot}$ can oxidize it directly (LeBel et al., 1992; Myhre et al., 2003; Ohashi et al., 2002). Transition metal ions or cellular haem proteins such as peroxidase must catalyze reaction with H_2O_2. Due to this broad spectrum of detected ROS and non-trivial reaction mechanism, the data should be interpreted more as a measure of cellular redox state than as intensity of any particular ROS production.

27

Probably due to this low specificity, there is quite an extensive debate about reliability of data obtained by this method (Halliwell et al., 2004). Many parameters have been proposed to influence the rate of H2DCFDA cleavage and oxidation, such as intracellular iron concentration (Tampo et al., 2003), cellular peroxidase level or haem protein content (LeBel et al., 1992; Ohashi et al., 2002). Furthermore, this probe is prone to photoactivation which may lead to overestimation of ROS production (Afzal et al., 2003; Bindokas et al., 2003).

1.5.2 Amplex Red

Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine) (Cohen et al., 1996; Zhou et al., 1997) is one of the numerous nonfluorescent compounds used in assays coupled with peroxidase (others are for example p-hydroxyphenylacetate (Hyslop et al., 1984) or homovanilic acid). In the presence of H_2O_2, these compounds are oxidized by HRP to fluorescent products (Boveris, 1984). The amount of H_2O_2 present is estimated by following the increase in fluorescence.

Both the cell inpermeability, as well as need for HRP, makes these probes suitable for measurement on isolated mitochondria or in the rare cases when extracellular H_2O_2 production is studied. Several precautions are required for accurate interpretation of reporter molecule test systems for detection of H_2O_2 in biological systems. Numerous biological compounds including thiols and ascorbate can serve as substrate for horseradish peroxidase (HRP) and thus compete with the detector molecule for oxidation, leading to underestimation of H_2O_2 formation. Competition with HRP by endogenous catalase for H_2O_2 can also lead to underestimation of H_2O_2 (Tarpey et al., 2004).

1.5.3 Dihydroethidium

Dihydroethidium (dihydroethidine, DHE) is frequently used as a probe for superoxide measurement. For quite a long time it was believed, that non-fluorescent dihydroethidium is by the action of O_2^{-} oxidized to a fluorescent ethidium. Recent work, however, suggests that the product is not ethidium, but rather hydroxy-ethidium (Zhao et al., 2003). Ethidium (or hydroxy-ethidium) tends to intercalate both into nuclear or mitochondrial DNA, which greatly enhances its fluorescence. Ethidium fluoresces strongly at around 600nm when excited at 500–530 nm.

The oxidation of DHE to ethidium was shown to be rapid when the oxidant was O_2^{-} but not when O_2, H_2O_2, HOCl or ONOO⁻ were used (Benov et al., 1998). However, DHE is

readily spontaneously oxidized and often gives a high background (Buxser et al., 1999). It can also undergo a direct redox reaction with ferricytochrome c (Benov et al., 1998). Another potential pitfall for measurements of mitochondrial ROS production is represented by the fact that oxidized ethidium has single positive charge. It is therefore readily uptaken by mitochondria and intercalated into mtDNA, which may lead to overestimation of mitochondrial $O_2^{\bullet-}$ production.

1.6 Mitochondrial diseases

Nothing is perfect in the world, neither mitochondria are - an estimated birth incidence for mitochondrial diseases is about 1:5000 (Thorburn, 2004). Mutations in genes coding for the broad spectrum of mitochondrial proteins lead to inborn errors of metabolism. The term mitochondrial diseases usually refers specifically to defects in OXPHOS apparatus, irrespective whether the cause is mutation in one of the OXPHOS subunits ("direct hit") or just "indirect hit" (i.e. depletion of mtDNA), which also affects OXPHOS complexes (Di Mauro, 2004).

Table 2: Classification of mutations responsible for mitochondrial diseases

Mutations in mtDNA	Mutations in ncDNA
Mutations in protein synthesis genes	*Mutations in respiratory chain subunits*
tRNA, rRNA mutations	complex I, complex II, complex III
Deletions, rearrangements	*Mutations in assembly proteins*
Mutations in protein coding genes	complex IV, complex III, complex V
LHON	*Defects of intergenomic signalling*
NARP/MILS	PEO with multiple Δ-mtDNA
	mtDNA depletion
	Defects of mitochondrial translation
	ribosomal subunit (MRPS16),
	translation elongation factor (EFG1)
	Defects of the lipid milieu
	Barth syndrome
	Defects of motility/fusion/fission
	Autosomal dominant optic atrophy

Often they are called mitochondrial encephalomyopathies, because of the prominent involvement of the nervous system and striated muscle. What makes research in this field both frustrating and fascinating is the fact, that these defects may have its origin both in nuclear genes as well as by mutations of mtDNA.

1.6.1 Mitochondrial diseases caused by mtDNA mutations

As a rule, mtDNA is transmitted maternally. Although recently the paternal transfer (from sperm mitochondria) of mtDNA was documented both in mouse (Gyllensten et al., 1991) and in human (Schwartz et al., 2002), there is general agreement that paternal transfer is rather very rare and insignificant exception than a rule (see (Schwartz et al., 2003) for review). Mutations in mtDNA therefore do not obey classical Mendelian genetics and are inherited maternally. Furthermore, there are $\sim 10^3$ copies of mtDNA in single mammalian cell. This brings into the scope also the level of heteroplasmy, i.e. ratio between mutated and non-mutated DNA.

The clinical manifestation of a pathogenic mtDNA will depend not only on its site within the mtDNA molecule but also on the proportion of mutant to wild-type molecules within cells. The threshold for biochemical expression may be around 60 % for mtDNA deletions (Hayashi et al., 1991) and up to 95 % for tRNA mutations (Chomyn et al., 1992), but we can also find mutations which have to be homoplasmic to express phenotype (LHON (Di Mauro, 2004)).

A striking feature of mtDNA disorders is their clinical heterogeneity, ranging from single-organ involvement to severe multisystem disease. When we consider the three main syndromes attributed to impaired mitochondrial protein synthesis: KSS (single, large scale mtDNA deletion), MELAS (typically, A3243G in tRNA^{Leu}) and MERRF (typically, A8344G in tRNA^{Lys}), we find the oxidative phosphorylation to be impaired in all of them to a similar extent; the clinical features, however, are clearly distinguishable.

Systematically, mtDNA mutations might be divided to point mutations and rearrangements (deletions and duplications, depletions). While point mutations are commonly maternally inherited, deletions and duplications (CPEO, KSS, and Pearson's syndrome) are most often sporadic or of unclear origin due to errors in mtDNA replication (see further). The mtDNA deletions face strong negative selection in rapidly dividing tissues (e.g. hematopoetic stem cells) whilst they accumulate in postmitotic tissues (muscle, brain) (Larsson et al., 1995).

The point mutations also split into two distinct groups, mutations in tRNA genes and mutations in structural genes.
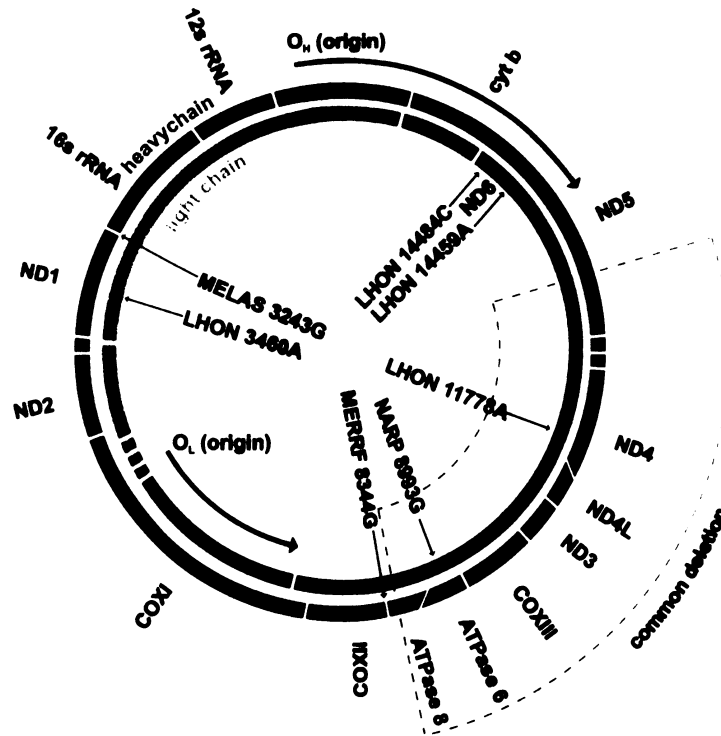

Figure 11: The human mitochondrial genome. The double helix can be separated into light and heavy chain. Light chain (orange) encodes tRNAs and one complex I subunit (ND6), while the heavy chain (green) encodes six complex I subunits (ND1, ND2, ND3, ND4L, ND4 and ND5), cytochrome *b* (cyt *b*), three complex IV subunits (COXI, COXII and COXIII), two ATP synthase subunits (ATPase 6, ATPase 8) and 14 tRNAs. Sites of most common point mutations are marked and the region of common deletion is outlined.

1.6.1.1 Mutations in tRNA genes

They disturb the whole process of mitochondrial proteosynthesis. Typical clinical manifestation includes abnormalities in CNS, myopathy, cardiomyopathy and lactic acidosis. The most common clinical manifestation of tRNA mutations are MELAS (typically, A3243G in tRNA^{Leu}) (Goto et al., 1990) and MERRF (typically, A8344G in tRNA^{Lys}) (Shoffner et al., 1990) syndromes. It is not surprising that at biochemical level, OXPHOS complexes with highest number of mitochondrially encoded subunits (complex I and complex IV) are among the most affected (Enriquez et al., 1995).

1.6.1.2 Mutations in structural genes

They are represented either as mutations in genes for complex I (ND1, ND4 and ND6) which lead to Leber's hereditary optic neuropathy (LHON) or by mutations in ATP6 subunit of complex V. Other mutations are much less frequent.

Leber's hereditary optic neuropathy classically manifests as acute-onset, bilateral, central vision loss. A total of 23 mtDNA missense mutations have been associated with LHON patients. However, detailed genetic analysis has revealed that only three 'primary' mutations (at positions 14459 (Jun et al., 1994; Shoffner et al., 1995), 3460 (Howell et al., 1995), and 14 448 (Johns et al., 1992; Mackey et al., 1992)) contribute in a major way to the development of blindness.

Second set of structural gene mutations is represented by mutations in ATP6 gene. In most patients mutation occurs at position 8993. Clinical symptoms depend on the level of heteroplasmy. At low levels (< 75%) it manifests as neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) while at high mutation loads close to homoplasmy there develops a maternally inherited Leigh syndrome (MILLS). The mutation leads to a reduction of ATP synthesis, although the amount of ATPase is not reduced. ATPase hydrolytic activity is normal (Houstek et al., 1999), but coupling of proton translocation and ADP phosphorylation within ATPase complex is affected. Three other mutations in ATPase6 at positions T8851C, T9176C and T9101C are associated with rather different phenotypes – bilateral striatal syndrome or Leigh syndrome (De Meirleir et al., 1995; Dionisi-Vici et al., 1998; Hartzog et al., 1993; Lamminen et al., 1995; Thyagarajan et al., 1995).

1.6.2 Mitochondrial diseases caused by ncDNA mutations

As documented in Table 2, mitochondrial diseases of nuclear origin are not restricted solely to mutations in OXPHOS subunits. Indeed the first described case was Autosomal dominant progressive external ophtalmoplegia (AdPEO) (Zeviani et al., 1989) characterized by the accumulation of multiple deletions of mtDNA in patient tissues. This belongs to the group of mutations affecting intergenomic signalling and replication of mtDNA. So far mutations in ANT1, Twinkle and mtDNA polymerase gamma have been found to be responsible for AdPEO. To the same category belong mtDNA depletion syndromes, where the cellular amount of mtDNA is dramatically decreased.

1.6.2.1 Mutations in structural genes

Isolated defects of individual OXPHOS complexes also split into two branches: mutations in structural subunits and mutations in assembly factors. Defects in structural proteins are most common in case of complex I, which is not that surprising when we consider its at least 39 nuclear-encoded subunits. Most often, these mutations manifest as early onset progressive neurological disorder, most often Leigh syndrome. So far there have been also described mutations in complex II genes (SDHA, SDHB, SDHC) and complex III (UQCRB), but one may expect that in future mutations in structural components of complex IV and V will be discovered.

1.6.2.2 Mutations in assembly factors

On the other hand, complex IV is a typical example of mutations in assembly factors. Quite common are mutations in SURF1 protein, but mutations in SCO1, SCO2, COX10 and COX15 were also described. In infancy the most frequent manifestation is Leigh syndrome, although other phenotypes, including cardiomyopathy and the leucodystrophy were reported as well. Mutations in assembly factors were found also in case of complex III (BCS1L).

Very recently, the first mutation in ATP 12, an assembler of mitochondrial ATP synthase, has been identified in single patient with lactic acidosis and progressive encephalopathy (De Meirleir et al., 2004). However, at present, more than dozen patients with ATP synthase deficiency have been diagnosed (Sperl et al., 2006) and in most of them mutations in *ATP11* and *ATP12* were excluded, and normal expression of the F_1-assembly genes was found. In some cases, new mutations in structural genes may be identified, but in others sequencing of structural genes did not reveal any mutation. Identification of new assembly proteins mutated in these individuals might therefore be expected. Most of these patients present with remarkably uniform clinical phenotype of early onset already in newborns, severe and often fatal hyperlactacidemia, hypertrophic cardiomyopathy and elevated levels of 3-methylglutaconic acid in urine.

1.6.2.3 Mutations in other genes related to oxidative phosphorylation

As our understanding of OXPHOS diseases in growing, new pathogenic mechanisms appear. Overview of them is in Table 2, here I give only very short summary. Apart from process of mitochondrial transcription, also mitochondrial translation may be affected. Mutation in mitochondrial mRNA binding protein LRPPRC was described (Mootha et al.,

2003) as well as mutations in two nuclear factors of the mitochondrial translation machinery: a homozygous mutation in MRPS16 (Miller et al., 2004), a protein of the small ribosomal subunit, and a homozygous mutation in EFG1 (Coenen et al., 2004), a mitochondrial translation elongation factor.

Furthermore, OXPHOS defects may arise as a result of coenzyme Q biosynthesis defect or due to mutation in Tafazzin, an acyltransferase responsible for transport of cardiolipin. Paraplegin, a mitochondrial metalloprotease, ABC7, an iron mitochondrial exporter or frataxin, protein putatively involved in iron handling might be just other examples of mitochondrial pathologies.

Finally, to further expand the spectrum of possible causes, mutations in mitochondrial fusion/fission apparatus have to be taken into account. So far, mutation in OPA1, a dynamin-related GTPase responsible for mitochondrial fusion process, was described (Delettre et al., 2000; Delettre et al., 2002). It has been implicated in autosomal dominant optic atrophy, an important cause of blindness in young adults.

1.6.3 Metabolic consequences and pathological mechanisms of mitochondrial diseases

1.6.3.1 Bioenergy deficiency

An inability of mitochondria to supply sufficient ATP to meet cellular needs is often assumed to be the primary effect of mitochondrial disease mutations. However, this may not be the only, or even the major, consequence of OXPHOS failure.

As apparent from inhibitor titration studies individual OXPHOS enzymes of mitochondrial energy metabolism can be inhibited to a certain extent without noticeable reduction of the mitochondrial coupled respiration rate. These threshold effects are different for individual OXPHOS complexes and they also display tissue specificity. For example in the case of ATPase, some 10 % of normal activity of the enzyme was found to be sufficient for 30–60 % functionality of the whole respiratory chain, depending on the type of tissue (Rossignol et al., 2003; Rossignol et al., 1999). This indicates that a significant decrease of ATPase capacity can be tolerated, at least under conditions when the energetic demands of the patient's organism are rather low. OXPHOS derived ATP may thus be essential only in specific cellular or physiological context, for example, in the heart under stress conditions (Cabrera et al., 2005).

1.6.3.2 Redox or metabolite imbalance

Many transport processes of mitochondria are directly driven by the electrochemical proton gradient of the inner mitochondrial membrane. This concerns not only the import and export of key metabolites but also of inorganic ions such as phosphate, sodium, potassium, or calcium (Nguyen et al., 2005; Szabo et al., 2005). In fact, significant portion of the electrochemical gradient is not utilized for ATP synthesis, but instead it is spent for fuelling of transport processes (Palmieri et al., 2000).

It is also important to take into account that OXPHOS activity plays a crucial role in the redox and substrate balance in the cell and, by extension, in the extracellular compartments. Inhibition of electron flow at the level of individual OXPHOS complexes results in the overoxidation of electron carriers downstream of the site of inhibition and over-reduction of upstream carriers. This may have a range of specific effects, for example, on nucleoside metabolism. Dihydroorotate dehydrogenase, one of the accessory enzymes of OXPHOS that donates electrons to ubiquinone, is required for pyrimidine biosynthesis. Severe disturbances of OXPHOS downstream of complex I thus render cells dependent on an exogenous supply of uridine (Gattermann et al., 2004).

1.6.3.3 Apoptotic mechanisms

Mitochondria are a key integration point of apoptotic signalling that influences a cell's death and survival (Green, 2005). In some instances, a functional OXPHOS system seems to be required for execution of programmed cell death, while in others OXPHOS dysfunction itself sensitizes cells to apoptosis or might even be its direct inducer. A more general view is that OXPHOS dysfunction leads to dysregulation of apoptotic signalling, so that some cells die inappropriately, whereas others, which have sustained different kinds of damage, do not undergo apoptosis, even if they should. This may lead to the failure of physiological functions and perhaps also favours the replicative senescence.

1.6.3.4 Elevated or disturbed production of ROS

The enhanced generation of ROS under conditions of respiratory chain blockade arises as a further pathogenic mechanism in mitochondrial diseases. For quite a long time

increase in ROS production was implicated in case of complex I deficiency as increased SOD activity was found in patient cells (Pitkanen et al., 1996). Further support was gained by observation, that specific inhibition of complex I and, to a lesser extent, complex III in intact cells results in a large increase in ROS production (Koopman et al., 2005).

Somehow different is the situation with deficiencies of ATP synthase. There is old observation that an exponential increase of mitochondrial ROS production occurs at high levels of $\Delta\Psi_m$ above 140 mV (Korshunov et al., 1997; Liu et al., 2002). Furthermore, decrease of $\Delta\Psi_m$ via stimulation of ATP synthase activity, a low ATP/ADP ratio, substrate limitation or increased proton permeability due to external or internal uncoupling lower the amount of ROS produced (Kadenbach, 2003). This seems to be relevant for cells with ATPase deficiencies. It has been demonstrated that both the cells with ATP6 mutations (Mattiazzi et al., 2004) and cells with nuclear defects of ATPase (Houstek et al., 1999; Mracek et al., 2006) maintain high values of $\Delta\Psi_m$ either in state 3-ADP or in state 4. In both types of ATP synthase disorders it was also documented that high $\Delta\Psi_m$ values really cause increased ROS production. The T8993G mutation caused an increase in MnSOD and ROS levels determined with fluorescent probe DCFDA (Geromel et al., 2001; Mattiazzi et al., 2004). Similarly, cells with quantitative defects of ATP synthase showed elevated levels of ROS and upregulation of antioxidative defence components (Mracek et al., 2006).

Sustained increase in ROS production may lead to subsequent damage of proteins (Stadtman et al., 2000), nucleic acids (LeDoux et al., 1999) and lipids (Rubbo et al., 1994) followed by apoptotic and necrotic processes in affected tissues (Sohal, 1991; Sohal, 1993). The high sensitivity to ROS damage of mitochondrial Fe-S cluster-containing enzymes, notably aconitase and OXPHOS complexes I-III themselves, may promote a catastrophic cycle of cellular damage under particular physiological conditions.

Last but not least, we should consider that the role played by ROS in mitochondrial pathogenesis might not be simple mechanistic link between increased production and increased damage to cells. Mitochondrial ROS were implied in cellular signalling and its dysregulation might be an important trigger for pathological processes. For example mitochondrial ROS production seems to be required for the normal induction of HIF (hypoxia-inducible factor), which is a master regulator of oxygen-sensitive gene expression in response to low oxygen levels (Brunelle et al., 2005; Guzy et al., 2005; Mansfield et al., 2005). Abnormalities in ROS production caused by OXPHOS dysfunction could thus result in aberrant HIF-mediated gene regulation. Such a mechanism has been suggested to underlie the inappropriate proliferative signalling associated with complex II

or fumarase (FH) deficiency, either of which promotes tumour formation (Eng et al., 2003; Pollard et al., 2003). On the contrary it must be admitted that there are studies indicating that tumourigenesis might proceed in these cases without any increase in ROS production (Pollard et al., 2005).

2 Aims of the thesis

From the broad field of mitochondrial ROS research we focused on individual sites of ROS production in mammalian mitochondria and on the role played by mitochondrial ROS in pathogenesis of mitochondrial diseases. The thesis had two main goals: (1) to characterize mammalian mGPDH as a potential ROS producer and (2) to elucidate putative role of ROS production in isolated OXPHOS defects, namely of ATP synthase. The specific aims were as follows:

1) mGPDH and ROS production

- Characterize mGPDH as a novel ROS producing site in mammalian mitochondria;
- Get insight into the reaction mechanism of ROS formation by this enzyme in comparison with other OXPHOS dehydrogenases;
- Describe kinetics of mGPDH turnover in liver after induction with thyroid hormones.

2) ROS and mitochondrial pathology

- Characterize patients with deficiency of ATPase of nuclear origin in terms of bioenergetics changes as well as changes in redox balance and ROS production;
- Study the involvement of ROS in pathogenesis of different types of mitochondrial diseases;
- Compare different methods for ROS measurement in intact cells and their applicability on cells with OXPHOS deficiencies.

3 Summary of the results

The thesis consists of 8 articles (6 published and 2 submitted), which may be divided into two major lines. The first (articles 1-4) deals with mitochondrial glycerophosphate dehydrogenase. We have shown that this enzyme is capable of massive ROS production and in the presented works we studied in more detail this ROS production as well as some aspects of enzyme biogenesis, so as to better understand its physiological significance. The second line (articles 5-8) represents our studies on the potential involvement of ROS in pathogenesis of mitochondrial diseases.

1. **Glycerophosphate-dependent hydrogen peroxide production by brown adipose tissue mitochondria and its activation by ferricyanide**; Drahota Z., Chowdhury SKR., Floryk D., Mráček T., Wilhelm J., Rauchová H., Lenaz G., Houštěk J.; *J Bioenerg Biomembr.* 2002; 34(2):105-13.

Indeed, this was first article to demonstrate ROS production by mammalian mGPDH. We studied oxidation of glycerophosphate by brown adipose tissue mitochondria, which are known to contain high amounts of mGPDH. We found significant GP-dependent AA-stimulated production of H_2O_2. To further verify our findings, we used three independent methods for H_2O_2 measurement – fluorescence detection with p-hydroxyphenylacetic acid, antimycine A - insensitive oxygen consumption, and luminometric detection. Both the facts that H_2O_2 production was increased by partial purification of mGPDH and that it was inhibited by polyborate, an inhibitor of mGPDH, may serve as a further proof, that mGPDH was the source of H_2O_2.

A drawback of this study was that for majority of experiments we used artificial one-electron acceptor, potassium ferricyanide, which highly stimulated H_2O_2 production. Some opponents thus argued that this does not necessarily reflect *in vivo* situation. Nevertheless, even this ferricyanide-induced H_2O_2 production was negligible with succinate or NADH as substrates. We concluded that mGPDH may act as H_2O_2 producer and that there must exist differences in the transport of reducing equivalents from mGPDH and SDH to the coenzyme Q pool.

2.   **Time-course of hormonal induction of mitochondrial glycerophosphate dehydrogenase biogenesis in rat liver**; Mráček T., Ješina P., Křiváková P., Bolehovská R., Červinková Z., Drahota Z., Houštěk J.; *Biochim Biophys Acta* 2005;1726(2):217-23

To continue our work, we looked for the system with inducible expression of mGPDH. Liver seemed to be good model as mGPDH is selectively upregulated upon injection of T_3 to euthyroid rats. This fact has been known for about 30 years (Lee et al., 1965), but to date there has not been detailed kinetic analysis of mGPDH induction. In our work we correlated the kinetics of the T_3 level in blood, the mRNA level in liver as well as the activity and amount of mGPDH in liver mitochondria after a single dose of T_3. Our studies documented relatively fast induction of the transcript (maximum after 12 hours) followed by slower increase of enzyme activity and content. Matching these profiles we documented, that the described increase in mGPDH activity is due to new biosynthesis of the enzyme and not a result of kinetic activation.

Taken together, we have clearly demonstrated that mGPDH is the only component of mammalian respiratory chain that is rapidly synthesized upon T_3 stimulus and displays fast turnover, which are characteristics essential for rapid "on demand" regulation according to different cellular needs. In other work from our laboratory the model of T_3 induction was used to demonstrate increase of ROS production in mitochondria from stimulated liver (Jesina et al., 2004), which further supported our data about mGPDH as a novel ROS producing site in mammalian mitochondria.

3.   **Specific Properties of Mitochondria from Human-term Placenta - Glycerophosphate-dependent Hydrogen Peroxide Production**; Honzík T., Drahota Z., Böhm M., Ješina P., Mráček T., Paul J., Zeman J., Houštěk J.; *Placenta* 2006; 27(4-5):348-56

Placenta is another transient tissue (together with brown adipose tissue of newborn mammals) with high activity of mGPDH. Originally we turned our attention to placenta because data in literature indicated, that activity of complex I is downregulated in placenta, which would provide us with an interesting model utilizing mGPDH for oxidation of cytosolic NADH. Contrary to this, we provided evidence, based on Western Blot quantification and enzyme activity measurements, that OXPHOS complexes are present in placenta at proportion similar to other tissues.

It is also the first work, where we used the newly established method for measurement of mitochondrial ROS production using fluorometric probe dichlorodihydrofluorescein diacetate. We found that placental mitochondria display high rate of glycerophosphate-dependent hydrogen peroxide production which was depressed by cytochrome *c*. mGPDH should therefore be considered as an additional source of ROS participating in induction of oxidative stress in placenta.

4. The contribution of mitochondrial respiratory chain components to the glycerophosphate dehydrogenase-dependent ROS production by brown fat mitochondria; Vrbacký M., Drahota Z., Ješina P., Mráček T., Vojtíšková A., Stopka P. and Houštěk J.; *Manuscript submitted*

In this study we focused on the differences between ROS production from individual OXPHOS dehydrogenases on already established model of brown adipose tissue mitochondria. We used two different ROS-sensitive fluorescent probes, CM-H$_2$DCFDA and Amplex Red, to determine the glycerophosphate- or succinate- dependent ROS production in mitochondria supplemented with respiratory chain inhibitors antimycin A and myxothiazol. We found that the ROS originating from dehydrogenase and coenzyme Q pool relative to ROS produced by complex III was 3-fold higher when oxidizing glycerophosphate than when oxidizing succinate. Furthermore, using both electron paramagnetic resonance (EPR) spectroscopy with DMPO as a spin trap as well as O$_2^{\cdot-}$-sensitive fluorescent probe dihydroethidium we demonstrated, that the primary ROS species produced by mGPDH is superoxide. Observed mGPDH-related O$_2^{\cdot}$ production was much higher than that related to SDH. We therefore concluded that there exist pronounced differences in the mechanism of ROS production originating from oxidation of glycerophosphate and succinate, indicating that electron transfer from mGPDH to coenzyme Q is highly prone to electron leak and superoxide generation.

5. Mitochondrial diseases and ATPase defects of nuclear origin; Houštěk J., Mráček T., Vojtíšková A., Zeman J.; *Biochim Biophys Acta* 2004; 1658(1-2):115-21

In this article we reviewed the current knowledge in the field of nuclear deficiencies of ATP synthase. We discussed two groups of patients. First is represented by the mtDNA mutations of ATP6 gene, including, homoplasmic mtDNA 2bp microdeletion 9205delTA, so far found in two cases. Although primarily this is mtDNA mutation, striking differences

in the biochemical and clinical presentations suggest an involvement of some so far unidentified nuclear-encoded factor that operates at the level of mitochondrial RNA processing.

The second group are *bone fide* nuclear mutations without mtDNA involvement. However, in most cases the underlying genetic mutation is unknown. Only in one case the mutation in *ATP12* assembly factor was described (De Meirleir et al., 2004). As we were unable to find any mutation in structural genes or assembly factors in all other cases that have been analysed, we postulate an involvement of another, yet unidentified ATPase assembly factor.

Furthermore, for the first time we discussed here an involvement of ROS in pathogenesis of nuclear ATPase deficiencies. This was documented in one of the patients by observed increase in ROS production, as measured by confocal microscopy.

6. Two Components in Pathogenic Mechanism of mitochondrial ATPase deficiency: Energy Deprivation and ROS Production; Mráček T., Pecina P., Vojtíšková A., Kalous M., Šebesta O., Houštěk J.; *Exp Gerontology* 2006; Mar 30 [Epub ahead of print]

In this article we further analyzed bioenergetics of cells with nuclear deficiency of ATP synthase. We used fibroblasts from four patients with ATPase activity decreased to 10-30% of controls. In these cells we found both defective energy provision as well as increase in ROS production. Mitochondrial ATP synthesis was decreased to 26-33% in patients cells and the titration of cellular respiration with aurovertin showed 6-fold decrease in I_{50}, meaning the ATPase threshold for coupled respiration is very low. In line with these findings, also the discharge of $\Delta\Psi_m$ was altered in patient cells. At state 3-ADP $\Delta\Psi_m$ was 20 mV higher than in control cells. Thus, the capacity for ATP production at conditions when energy demands are increased, is very likely to be insufficient in patient cells.

But not only the ATP production is defective in these cells - in intact cells we found 2-fold increase in ROS production in patients as compared to controls. ROS production rate was sensitive to uncoupler (FCCP) and thus apparently related to increased $\Delta\Psi_m$ in patient cells. Interplay of both components (energy deprivation and oxidative stress) interconnected via the $\Delta\Psi_m$ thus seems to be essential for understanding of the differences the phenotypic presentation of ATPase deficiencies.

7. **Mitochondrial diseases and genetic defects of ATP synthase**; Houštěk J., Pícková A., Vojtíšková A., Mráček T., Pecina P., Ješina P.; *BBA – Bioenergetics* 2006; Apr 19 [Epub ahead of print]

Here we discussed the current knowledge about assembly of ATPase and its defects. Alteration of ATPase assembly may cause two types of isolated defects: qualitative and quantitative. In qualitative ones the enzyme is structurally modified and does not function properly, while in quantitative ones there are insufficient amounts of otherwise structurally normal enzyme. We compared our data obtained from patients with ATPase defects with those obtained in other laboratories. We also proposed that new primary genetic cause will be identified as most of the current patients do not bear currently known mutations.

8. **Comparison of various methods for measurement of mitochondrial reactive oxygen species production in fibroblasts from patients with ATP synthase deficiency using CM-H₂DCFDA**; Mráček T., Šebesta O., Vojtíšková A., Wieckowski M.R., Rizzuto R., Houštěk J.; *Manuscript submitted*

Our research on ROS production brought us also to some doubts about methodological aspects of ROS measurement as the methods used are often criticized to give conflicting results. We established three different approaches for measurement of ROS production with CM-H₂DCFDA probe: plate-reader, single- and two-photon excitation confocal microscopy. For microscopic measurements we also related the signal to mitochondrial mass, as determined by mitochondria selective probe MitoTracker DeepRed. We tried to compare data obtained by these three methods with respect to absolute and relative values of ROS production and the intensity of probe autooxidation and photoactivation. All three methods detected the difference between control and patient cells. Quantification of the data, however, revealed differences, most probably due to photoactivation.

We introduced the measurement of FCCP sensitive portion of ROS production, which should represent the mitochondria specific component. Evaluating this component helps in eliminating of unspecific DCFDA oxidation and even to some extent eliminates the effect of photoactivation. We also suggested usage of the Δ FCCP$_{patient}$ / Δ FCCP$_{control}$ ratio which appeared to be very promising for quantification of differences in mitochondrial ROS production among individual cell lines.

In conclusion, all methods proved to be plausible for ROS measurements on intact cells with superior sensitivity being the main advantage of microscopy and high-throughput screening of plate-reader.

4 Conclusions

From our results on mGPDH we conclude that:

- We have defined mGPDH as a new site of ROS production in mammalian mitochondria.
- Our experiments indicate that $O_2^{\bullet-}$ is the primary radical formed by mGPDH. It presumably originates from $Q^{\bullet-}$ formed as intermediate by mGPDH.
- The high rate of ROS production from mGPDH in comparison with other dehydrogenases may reflect the simple structure of mGPDH, that appears to be low in FeS content and lacks additional subunits interacting with coenzyme Q. Low affinity towards coenzyme Q may therefore be another explanation.
- The turnover of mGPDH is relatively fast and regulated. It responds very fast to thyroid induction and enzyme half-life suggests also for selective degradation.

Data on patients with mitochondrial diseases lead to the following conclusions:

- The measurement of *in vivo* ROS production clearly demonstrated enhanced levels of oxidative stress in fibroblasts of patients with deficiency of ATP synthase.
- Intensity of ROS production is sensitive even to mild uncoupling with FCCP. High steady-state levels of $\Delta\Psi_m$ in these patients might therefore be responsible for oxidative stress.
- By comparison of data from various types of OXPHOS deficiencies we suggest, that increased ROS seems to be systemic feature only in case of C V defects.

5 Epilogue or (if you like it) an informal view of the research done

"If it be true that good wine needs no bush, 'tis true that a good play needs no epilogue. Yet to good wine they do use good bushes; and good plays prove the better by the help of good epilogues."

Says Rosalind in W. Shakespeare's *As You Like It*. Allow me therefore to present you at this place the less formal but more extent summary of our work.

Mitochondrial glycerophosphate dehydrogenase

We began our work by recognition of the mGPDH capability to produce hydrogen peroxide. It took several years before mGPDH has become wider accepted as a new ROS producing site in mammalian mitochondria. Although now there is agreement on this fact, the molecular mechanism still remains elusive. Brand's group studied mGPDH in Drosophila mitochondria, where they also found significant levels of GP dependent ROS production. Based on the experiments with exogenous superoxide dismutase they proposed that the primary radical produced is $O_2^{\cdot-}$ (Miwa et al., 2005; Miwa et al., 2003) and it was definitely confirmed by our so far unpublished experiments using EPR spectroscopy. This speaks for the semiquinone as a source of ROS and further supports our originally proposed idea of low binding affinity for Q as a main reason for electron leak on mGPDH.

Mitochondrial diseases and ROS production

Reactive oxygen species were implied as potential pathogenic factor in OXPOS diseases already some time ago. However, the evidence for this theory was largely indirect or speculative. The mGPDH looked very attractive target in this field. Its expression is highly tissue specific and its upregulation may be expected under conditions where glycolytic NADH needs to be utilized. Although these thoughts brought us to studies of ROS production in OXPHOS patients, so far we were not able to find proof for them and it is questionable, whether we would ever be.

Most of our work we did on patients with deficiency of ATPase. Here we could clearly document marked increase in ROS production as well as defective ATP production. This led us to proposition of "two component" pathology, where both the insufficient

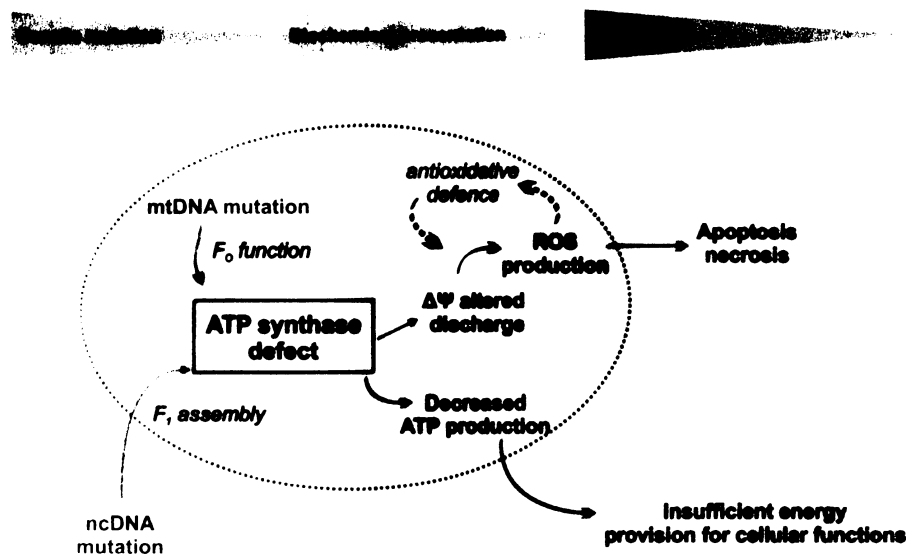provision of ATP and ROS production contribute to the pathologic presentation of the disease (See Figure 12).

Figure 12: Pathogenic mechanism of ATP synthase defects - energy deprivation and increased ROS production.

What about mutations in other complexes? Throughout the years we also analyzed cells with defects in complex I (NDUFS1, NDUFS3, NDUFS4 and three unknown nuclear defects) and complex IV (SURF1). The comparison is shown in Figure 13. It is apparent that most pronounced increase is found in case of complex V patients, complex I patients are highly variable, with documented increases in some cases and without changes in others and complex IV patients show no increase in ROS production at all. Also results of other groups on complex I (Iuso et al., 2006) or complex V (Geromel et al., 2001; Mattiazzi et al., 2004) seem to support these measurements. Summed up, it seems that probably the most important regulator of mitochondrial ROS production in intact cells is $\Delta\Psi_m$ and its defective dissipation leads to increased oxidative stress.

Somehow different is the case of complex I defects. Here it seems that some of the mutations affect electron transport in that way, that (unphysiological) leak may occur, while the others, although affecting CI activity, do not allow for electron leak. Not so surprising is also the result on Complex IV cells. Inability to shuttle electrons to CIV will actually prevent ROS formation on CIII and defective built-up of $\Delta\Psi_m$ on the other hand prevents reverse electron flow and ROS production on CI.
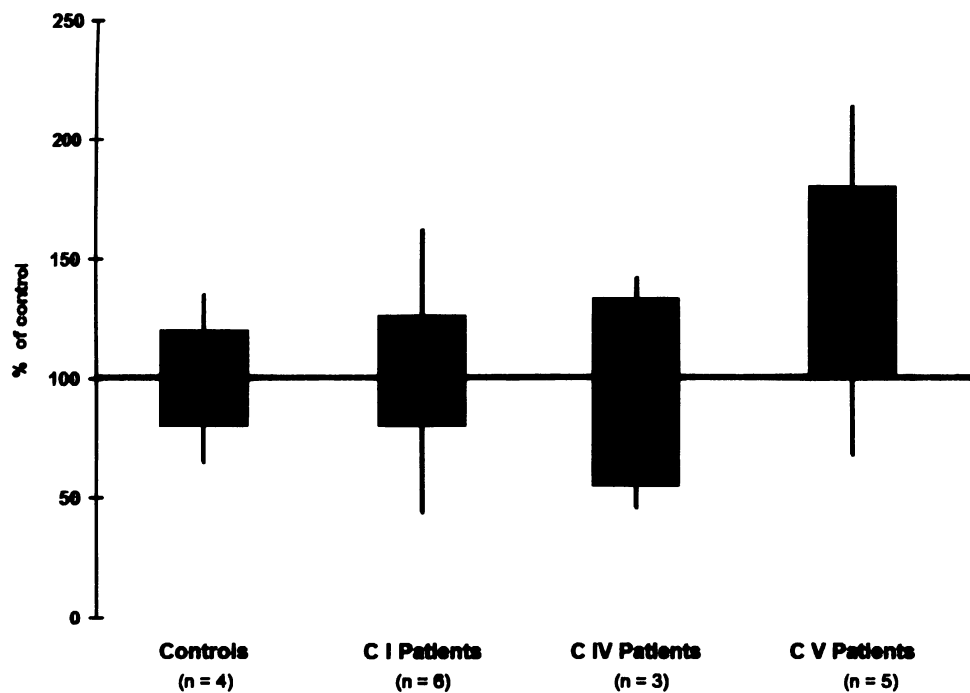
Figure 13: ROS production in different OXPHOS deficiencies.

So, the situation is becoming clearer than it was several years ago. Results of different groups begin to match together and some physiological consequences may already be drawn. But in any case, there is still much work ahead of us, before we will understand all aspects of mitochondrial ROS biology.

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Article 1

Glycerophosphate-Dependent Hydrogen Peroxide Production by Brown Adipose Tissue Mitochondria and Its Activation by Ferricyanide

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Oxidation of glycerophosphate (GP) by brown adipose tissue mitochondria in the presence of antimycin A was found to be accompanied by significant production of hydrogen peroxide. GP-dependent hydrogen peroxide production could be detected by *p*-hydroxyphenylacetate fluorescence changes or as an antimycin A-insensitive oxygen consumption. One-electron acceptor, potassium ferricyanide, highly stimulated the rate of GP-dependent antimycin A-insensitive oxygen uptake, which was prevented by inhibitors of mitochondrial GP dehydrogenase (mGPDH) or by coenzyme Q(CoQ). GP-dependent ferricyanide-induced peroxide production was also determined luminometrically, using mitochondria or partially purified mGPDH. Ferricyanide-induced peroxide production was negligible, when succinate or NADH was used as a substrate. These results indicate that hydrogen peroxide is produced directly by mGPDH and reflect the differences in the transport of reducing equivalents from mGPDH and succinate dehydrogenase to the CoQ pool. The data suggest that more intensive production of reactive oxygen species may be present in mammalian cells with active mGPDH.

KEY WORDS: Brown adipose tissue; mitochondrial glycerophosphate dehydrogenase; ferricyanide; hydrogen peroxide.

INTRODUCTION

Reactive oxygen species (ROS) are proposed as mediators of tissue injury in several human pathologies (Halliwell, 1987; Richter, 1997; Schapira, 1994), in acceleration of degenerative processes in ageing (Barja, 1999; Barja and Herrero, 1998; Sohal, 1993), and in initiation of necrotic and apoptotic processes (Kroemer *et al.*, 1998; Mignotte and Vayssiere, 1998; Pedersen, 1999). Most of ROS are produced in mitochondria because of interaction

of oxygen with free electrons released by the respiratory chain. The leak of electrons from the mitochondrial respiratory chain has been localized in Complex I (NADH-CoQ reductase) and Complex III (CoQ-cytochrome *c* reductase) (Herrero and Barja, 2000; Kwong and Sohal, 1998; Sohal, 1991; Turrens *et al.*, 1986). Under physiological conditions, toxic effects of ROS formed from released electrons are compensated for by the cell antioxidative defense mechanisms, which decompose the ROS produced. As suggested recently, another defense mechanism may involve the function of uncoupling proteins, which decrease ROS production by lowering the inner mitochondrial membrane potential (Negre-Salvayre *et al.*, 1997). However, formation of ROS may be highly activated

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Key to abbreviations: BAT – brown adipose tissue, CoQ – coenzyme Q, SUC – succinate, GP – glycerophosphate, mGPDH – mitochondrial glycerophosphate dehydrogenase, ROS – reactive oxygen species, HRP – horse-radish peroxidase.

under pathological situations, e.g., during hypoxic injury or because of the respiratory chain defects in mitochondrial encephalomyopathies (Wallace, 1999) which increase the reducing potential of the electron carriers of the respiratory chain towards the NADH.

The high rate of ROS production has been also detected in insect fly muscle mitochondria (Bolter and Chefurka, 1990; Sohal, 1991) and in brown adipose tissue (BAT) mitochondria (Sekhar et al., 1987), when glycerophosphate (GP) was used as a respiratory substrate. It was suggested that mitochondrial GP dehydrogenase (mGPDH) could be another complex of the mitochondrial respiratory chain where the leak of electrons may occur.

mGPDH is involved in oxidation of cytosolic NADH by GP shuttle which bypasses Complex I (Bucher and Klingenberg, 1958), in regulation of triglyceride synthesis (Kornacker and Ball, 1968) and possibly also in uncoupling protein-independent energy dissipation which involves also malic enzyme (Bobyleva et al., 1993, 2000). The highest activity of mGPDH in equimolar proportion with cytosolic GPDH was found in BAT (Houstek et al., 1975). However, it is quite evident that this enzyme plays an important role also in other tissues and cells. mGPDH was studied in pancreatic beta cells (Ishihara et al., 1996; Meglasson et al., 1989), brain (Cottingham and Ragan, 1980a,b), heart muscle (Scholz et al., 1997), placenta (Swierczynski et al., 1976), testis (MacDonald and Brown, 1996), fibroblasts (Chretien et al., 1994), and after hormonal induction also in liver (Bobyleva et al., 2000). However, the multiple roles of mGPDH in cellular metabolism are not yet fully clarified, and production of ROS could be another important feature of this enzyme.

In this paper we present the evidence that mGPDH is very potent producer of hydrogen peroxide in BAT cells and that this hydrogen peroxide production may be highly increased when the electron flow from the enzyme to CoQ pool is affected by the one-electron acceptor, potassium ferricyanide. This also indicates differences in electron transport from mGPDH and succinate dehydrogenase to the CoQ pool.

MATERIALS AND METHODS

Isolation of Brown Adipose Tissue Mitochondria

Mitochondria were isolated from BAT of adult male Syrian hamsters, cold adapted at 4°C for 3 weeks as described by Hittelman et al. (1969). Isolation medium was 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. For experiments freshly isolated mitochondria or mitochondria frozen at −70°C were used.

Fluorometric, Polarographic, and Luminometric Detection of Hydrogen Peroxide Production

Hydrogen peroxide formation was detected fluorometrically according to Hyslop and Sklar (1984) by following the oxidation of p-hydroxyphenylacetate into a fluorescent form during the enzymatic reduction of hydrogen peroxide by horse-radish peroxidase (HRP). The reaction mixture contained 154 mM KCl, 5 mM K_3PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4 and 160 μg p-hydroxyphenylacetate/mL, 80 units of HRP/mL, and 80 μg of mitochondrial protein/mL. The rate of hydrogen peroxide production was followed using Perkin Elmer LS-5 spectrofluorometer (excitation 317 nm, emission 400 nm). Known concentrations of hydrogen peroxide were used to establish the standard concentration curve.

Oxygen consumption was measured using High Resolution Oxygraph from OROBOROS, Austria (Gnaiger et al., 1995). Measurements were performed at 30°C in 1.5 mL of incubation medium containing 100 mM KCl, 10 mM Tris-HCl, 4 mM K_3PO_4, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.2, using 0.1–0.5 mg/mL of the mitochondrial protein. OROBOROS software was used for calculations of oxygen production and for graphic presentation of experimental data. Oxygraphic curves represent the first derivation of oxygen tension changes, the area of peaks represents the total oxygen consumption, and the height of the peak represents the rate of reaction.

Luminescence was measured by Luminometer 1250 from Bioorbit (Finland) as described earlier (Wilhelm and Vilim, 1986). Measurements were performed at room temperature in 1 mL of medium containing 50 mM KCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.4, and 0.1–0.3 mg of mitochondrial protein. Luminol (5-amino-2,3-dihydro-1,4-phtalazionedione) concentration was 1 mM, and HRP was 2.5 units/mL. The reaction was started by addition of 100 μl of 5 mM potassium ferricyanide. A calibration curve of hydrogen-peroxide-induced luminescence was determined under the same experimental conditions. The luminescence peak reached maximum values within 2/3 s after addition of potassium ferricyanide. For evaluation of peroxide production the maximum value (peak) was used. The peak value was found to be fully proportional to luminescence peak area.

Solubilization and Purification of mGPDH

Frozen–thawed mitochondria were washed twice in 10 mM K-phosphate buffer, pH 7.4 by 10 min centrifugation at 10,000 g, and the protein was adjusted to 10 mg/mL. Mitochondria were solubilized by digitonin treatment as

described by Klement *et al.* (1995). An equal volume of 10 mg digitonin/mL dissolved in 10 mM *K*-phosphate buffer was added to the mitochondrial suspension and the mixture was incubated for 15 min on ice. Unsolubilized material was removed by centrifugation for 30 min at 30,000 g at 4°C. Supernatant (maximum 20 mg protein) was applied on the hydroxylapatite column (Biogel HTP, BioRad, 10 cm × 1 cm) equilibrated with 10 mM *K*-phosphate buffer pH 7.4. The same buffer was used for elution of unbound proteins. Fractions containing the highest activity of mGPDH were collected and concentrated with Amicon PM-30 membrane. This partially purified enzyme was used for enzyme activity measurements and for detection of hydrogen peroxide production. Enzyme activity was measured spectrophotometrically according to Garrib and McMurray (1984). The SDS polyacrylamide gel electrophoresis (Schagger and von Jagow, 1987) and the Blue-Native electrophoresis (Schagger and von Jagow, 1991) were used for determination of purity of the soluble enzyme preparation.

Chemicals

Mercaptodicarbanonaborate (Na.5-SH, 7,8-$C_2B_9H_{11}$) was synthesized by Dr J. Plesek in the Institute of Inorganic Chemistry, Academy of Sciences of the Czech Republic, Prague. D,L-glycerol-3-phosphate, *p*-hydroxymercuribenzoate, *p*-hydroxyphenylacetate, and antimycin A were from Sigma, and catalase was from Boehringer (Germany). All other chemicals were from Lachema (Czech Republic).

RESULTS

Glycerophosphate-Dependent Hydrogen Peroxide Production by Brown Adipose Tissue Mitochondria

In agreement with the study of Sekhar *et al.* (1987), we found, using fluorometric detection, that BAT mitochondria generate 3.1 ± 0.05 (*n* = 10) nmol of H_2O_2/min/mg protein when GP was used as a substrate and 1.74 nmol with succinate (Fig. 1). GP-dependent hydrogen peroxide formation was completely abolished by 0.1 mM mercaptodicarbanonaborate (Fig. 1) which strongly inhibits mGPDH activity (Drahota *et al.*, 1995) or with 0.5 mM *p*-hydroxymercuribenzoate (not shown), another inhibitor of mGPDH (Rauchova *et al.*, 1985).

Hydrogen peroxide formation could be also detected by polarographic determination of GP-dependent oxygen consumption insensitive to antimycin A or KCN. Similarly as in fluorometric measurements we found
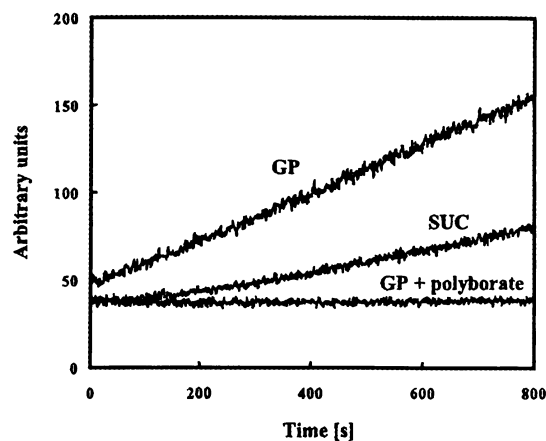

Fig. 1. Fluorometric detection of hydrogen peroxide formation by BAT mitochondria. Measurements were performed in the presence of 2 μM antimycin A and 25 mM glycerophosphate (GP) or 25 mM succinate (SUC) using frozen–thawed BAT mitochondria. Where indicated, 0.1 mM mercaptodicarbanonaborate was added 30 s prior to addition of glycerophosphate (GP + polyborate).

almost twofold higher rate of hydrogen peroxide production when GP was used as a substrate than in the presence of succinate (Fig. 2). In accordance with previous studies (Houstek *et al.*, 1978) the activity of succinate cytochrome *c* reductase was even higher than that of GP cytochrome *c* reductase in BAT mitochondria (0.365 ± 0.034 and 0.15 ± 0.016 μmol/min/mg protein, respectively). This indicates, that in the presence of antimycin A more hydrogen peroxide is produced during oxidation of GP than during oxidation of succinate.

Ferricyanide-Induced Hydrogen Peroxide Production

The rate of antimycin A-insensitive oxygen consumption was highly accelerated by the addition of potassium ferricyanide (Fig. 3), a one-electron acceptor of mitochondrial dehydrogenases (Klingenberg, 1970). Ferricyanide-induced GP-dependent oxygen consumption could be demonstrated with freshly isolated (Fig. 3) as well as with frozen–thawed mitochondria.

As shown in Fig. 3, when the added potassium ferricyanide was reduced by mGPDH, the rate of oxygen consumption returned to the original low values. Subsequent additions of potassium ferricyanide again activated the rate of oxygen consumption. In contrast, succinate- or NADH-induced antimycin A-insensitive oxygen consumption measured in frozen–thawed mitochondria was lower than that with GP and was not further enhanced by ferricyanide.
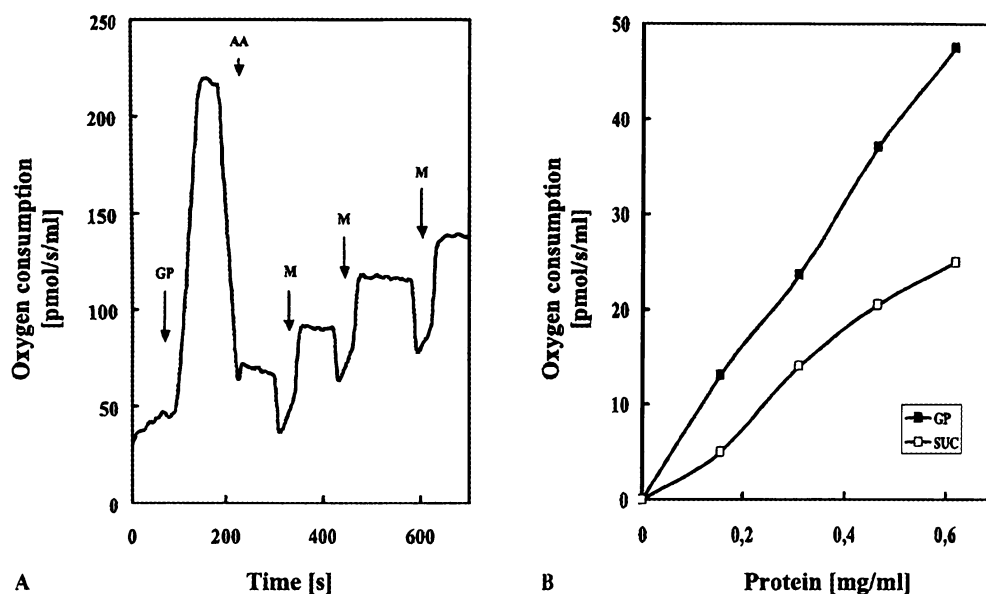
Fig. 2. Antimycin A-insensitive oxygen uptake in the presence of glycerophosphate (GP) and succinate (SUC). (A) Representative recording of oxygen consumption in the presence of glycerophosphate. (B) Calculated antimycin A-insensitive oxygen consumption in the presence of glycerophosphate and succinate. Frozen BAT mitochondria (0.15 mg/mL) were suspended in KCl medium (see Methods). Where indicated, 10 mM glycerol phosphate or succinate was added. Oxygen consumption was inhibited by 2 μM antimycin A (AA) followed by three subsequent additions of 0.15 mg/mL of mitochondria (M).
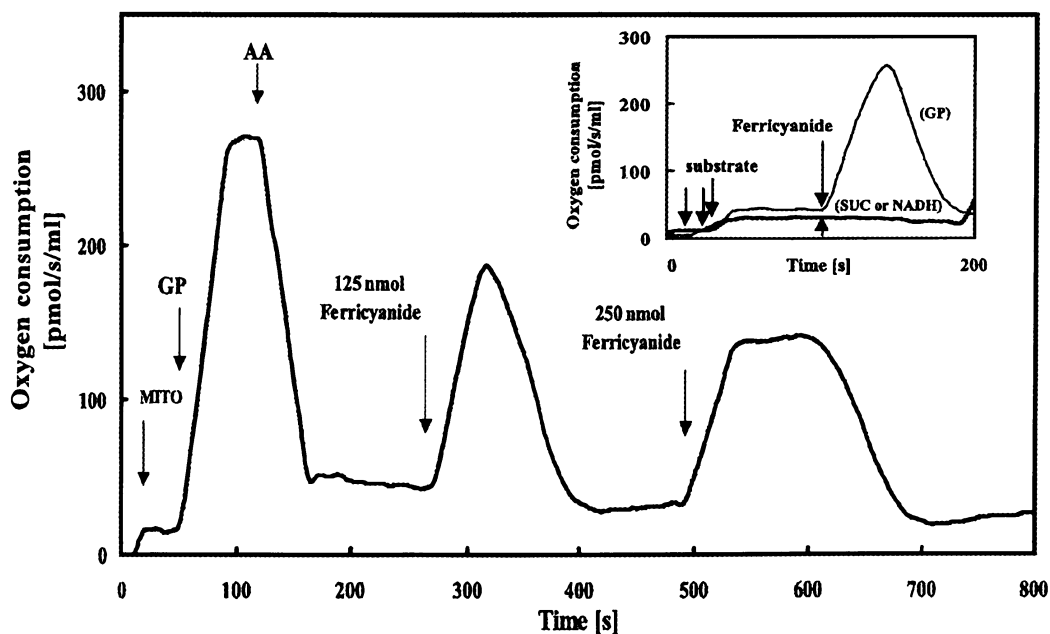
Fig. 3. Ferricyanide-induced, glycerophosphate-dependent, antimycin A-insensitive oxygen consumption by BAT mitochondria. For experiment, 0.3 mg protein/mL of freshly isolated BAT mitochondria (MITO), 10 mM glycerophosphate (GP), 2 μM antimycin A (AA), and 125 and 250 nmol of ferricyanide were used. Insert shows the effect of ferricyanide on antimycin A-insensitive oxygen consumption in the presence of 10 mM glycerophosphate or 10 mM succinate or 0.1 mM NADH using frozen–thawed BAT mitochondria.

The rate of the ferricyanide-induced oxygen consumption (height of the peak) was dependent on the amount of mitochondria used, and the total amount of oxygen consumed (area of the peak) was proportional to the amount of ferricyanide added. In Fig. 3 the total oxygen consumption was 42.3 nAt O and 61.2 nAt O, respectively using 125 and 250 nmol ferricyanide. The stoichiometry between the added potassium ferricyanide and the oxygen consumed at low ferricyanide concentrations (80–150 μM) was about 0.3 nAt O/nmol ferricyanide. At higher ferricyanide concentrations (500–1000 μM) or after repeated additions of potassium ferricyanide the ratio decreased to values about 0.2 and lower (Table I). This indicates that during the transfer of electrons from mGPDH to the one-electron acceptor, hydrogen peroxide was produced.

Inhibition of Hydrogen Peroxide Production by Catalase, mGPDH Inhibitors, and CoQ

Hydrogen peroxide production by BAT mitochondria oxidizing GP in the presence of antimycin A and ferricyanide could be also confirmed by the effect of catalase. As shown in Fig. 4, the total oxygen consumption

Table I. GP-Dependent, Potassium Ferricyanide-Induced Hydrogen Peroxide Production: Ratio Between Added Potassium Ferricyanide and Oxygen Consumed

Ferricyanide added (nmol)	Oxygen consumed (nAt)	Oxygen/ferricyanide ratio (nAt O/nmol ferricyanide)
125	37 ± 1.7	0.30 ± 0.012
250	71 ± 1.5	0.28 ± 0.010
500	105 ± 1.8	0.21 ± 0.019
1000	162 ± 2.2	0.16 ± 0.023

Note. Oxygen uptake was measured in the presence of 20 mM glycerophosphate and 2 μM antimycin A. Frozen–thawed mitochondria were used. Data indicate mean ± SD, $n = 3$.

after addition of ferricyanide was 54.7 nAt O. The release of oxygen after addition of catalase was 20.5 nAt O. Subsequent addition of potassium ferricyanide in the presence of catalase induced oxygen consumption that was also about 50% lower than that in the absence of catalase (28.0 n At O).

Similarly, as in fluorometric measurements, potassium-ferricyanide-induced hydrogen peroxide production was completely abolished by the inhibition of mGPDH by mercaptodicarbanonaborate or oleate, another inhibitor of mGPDH (Houstek and Drahota,
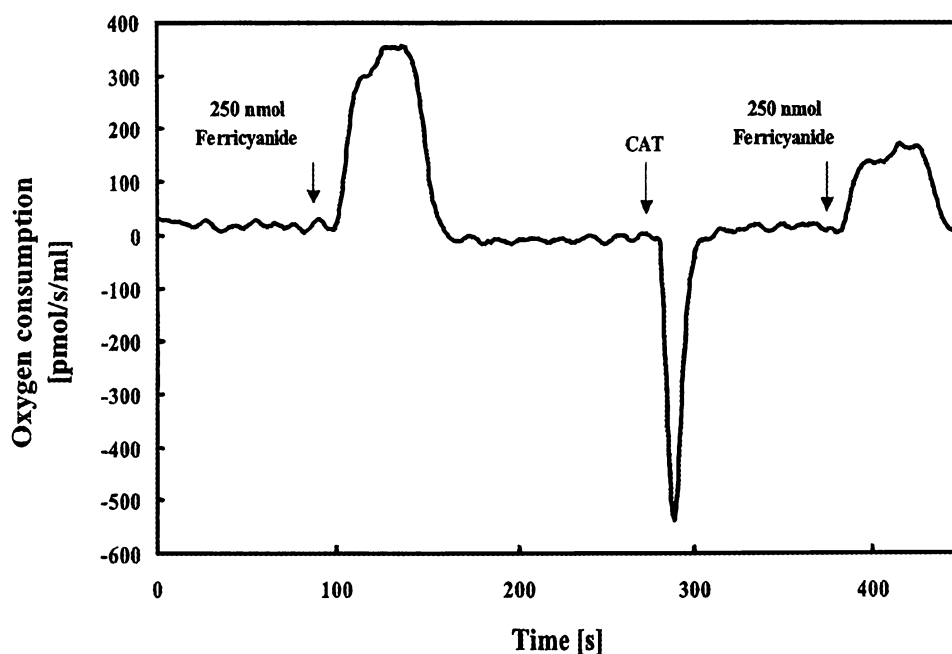

Fig. 4. The effect of catalase on ferricyanide-induced hydrogen peroxide production. Measurements were performed in the presence of 10 mM glycerophosphate and 2 μM antimycin A using frozen–thawed BAT mitochondria. Where indicated, 250 nmol of ferricyanide and 4300 U/mL of catalase (CAT) were added.

Table II. Effect of mGPDH Inhibitors on GP-Dependent, Potassium Ferricyanide-Induced Oxygen Consumption

Additions	Oxygen consumed (nAt/min/mg protein)	%
GP + KCN	5.20 ± 0.06	9
GP + KCN + 125 nmol ferricyanide	59.26 ± 0.15	100
GP + KCN + 40 μM C₂B₉SH + 125 nmol ferricyanide	7.04 ± 0.11	12
GP + KCN + 2 μmol oleate/mg protein + 125 nmol ferricyanide	14.46 ± 0.05	24

Note. Oxygen consumption was measured in frozen–thawed mitochondria (0.3 mg protein/mL) in the presence of 10 mM GP and 0.5 mM KCN. Where indicated potassium ferricyanide, sodium mercaptodicarbanonaborate (C_2B_9SH) or sodium oleate were added. Data indicate mean ± SD, $n = 4$.

1975) (see Table II). GP-dependent ferricyanide-induced hydrogen peroxide production was also abolished by addition of CoQ_3 (Fig. 5). At 10 μM CoQ_3 the total oxygen consumption was decreased from 40.0 to 9.4 nAt O,

respectively. As shown in Fig. 5, the half-maximal inhibition was obtained at 5 μM CoQ_3.

Luminometric Detection of Glycerophosphate-Dependent Hydrogen Peroxide Production by Mitochondria and Partially Purified mGPDH

As demonstrated in Fig. 6, in the presence of GP, intensive ferricyanide-induced peroxide production can be also detected by luminometry. Luminometric detection showed the same kinetics and the same substrate dependency as polarographic measurements. We obtained a small, but significant luminescence signal when succinate was used (Fig. 6). Evidently, luminometry is more sensitive in detection of small changes in hydrogen peroxide production than polarography. With NADH no luminescence signal was obtained (not shown). Similarly as in polarographic measurement, mGPDH inhibitors and CoQ_3 inhibited hydrogen peroxide formation (not shown).
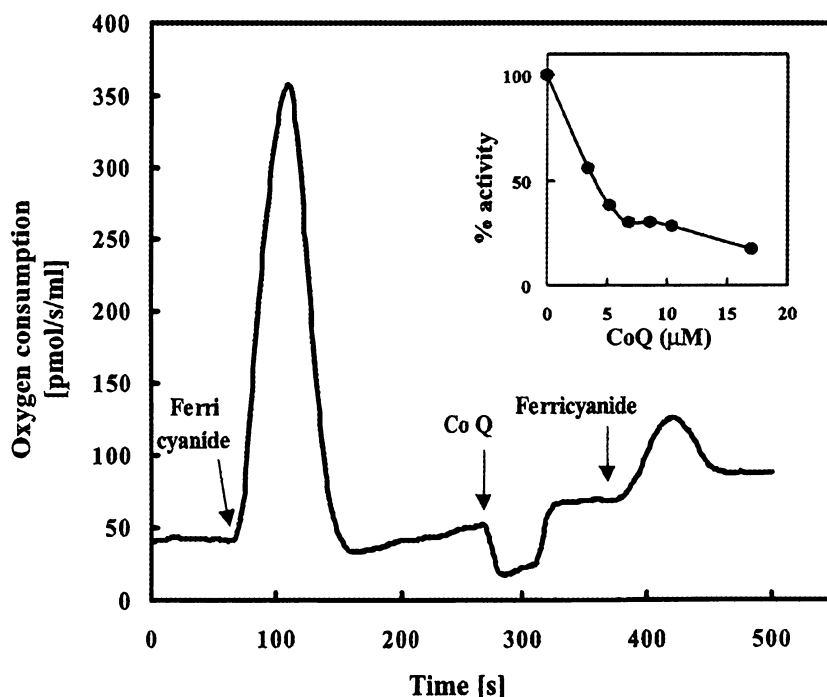

Fig. 5. Inhibition of ferricyanide-induced, GP-dependent hydrogen peroxide production by Coenzyme Q. Measurements were performed in the presence of 10 mM glycerophosphate and 2 μM antimycin A using frozen–thawed BAT mitochondria. Where indicated, 125 nmol of ferricyanide and 10 μM Coenzyme Q_3 (CoQ) were added. Insert shows the inhibitory effect of 2.5–17.5 μM concentrations of CoQ.
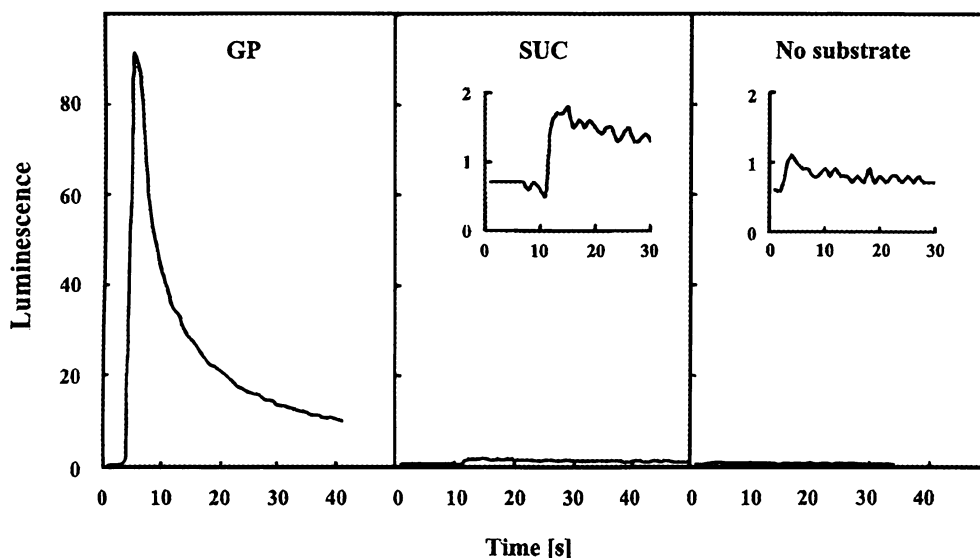
Fig. 6. Luminescence detection of ferricyanide-induced hydrogen peroxide production by BAT mitochondria in the presence of 10 mM glycerophosphate or 10 mM succinate (SUC). A total of 0.2 mg of mitochondrial protein was used. The same results were obtained with freshly isolated and with frozen–thawed mitochondria.

In further experiments we tested the ability of mGPDH to catalyze hydrogen peroxide production, using partially purified mGPDH from BAT mitochondria. Based on electrophoretic analysis and enzyme activity measurements, the enzyme was purified 20-fold. The enzyme preparation was free of contamination by succinate and NADH dehydrogenases, cytochrome c oxidase, and bc_1 complex. Further purification was accompanied by rapid loss of the enzyme activity, as also described by Cole et al. (1978).

As shown in Fig. 7, isolated mGPDH was able to catalyze the ferricyanide-induced, GP-dependent production of hydrogen peroxide. In comparison with hydrogen peroxide production by BAT mitochondria, luminescence signal elicited by the isolated mGPDH was about 10-fold higher when calculated per mg protein. Hydrogen peroxide production by the isolated mGPDH was also proportional to the amount of protein added in the range up to 10 μg/mL. Similarly as in isolated mitochondria, the hydrogen peroxide production was completely inhibited by mGPDH inhibitors and by CoQ3 (not shown).

DISCUSSION

Our results clearly showed that mGPDH represents an additional site of ROS generation in the mammalian mitochondrial respiratory chain. Our data on isolated enzyme further proved previous suggestions (Bolter and

Chefurka, 1990) that mGPDH can act as ROS generator. This could be especially important for mammalian cells and tissues with high activity of this FAD-dependent dehydrogenase, namely in BAT of newborn mammals and hibernators (Houstek et al., 1975, 1978), pancreatic beta cells (MacDonald et al., 1996), or brain (Cottingham and Ragan, 1980a,b). However, the mechanism of GP-dependent hydrogen peroxide formation by mGPDH is not yet quite clear.

Hydrogen peroxide may be formed in analogy with mechanism proposed for ROS production by Complex I and III from ubisemiquinone radical III (Barja and Herrero, 1998; Kwong and Sohal, 1998; Turrens et al., 1986). Our data, however, indicate that there exist differences in the transport of reducing equivalents from mGPDH and succinate dehydrogenase to the CoQ pool. GP-induced ROS production is higher than that induced by succinate. This difference could be due to the fact that whereas a specific CoQ-binding protein was detected in succinate dehydrogenase (succinate CoQ-reductase) (Yu et al., 1978), it appears to be missing in mGPDH (Cottingham and Ragan, 1980a,b; Rauchova et al., 1992). This CoQ-binding protein evidently represents a natural protection of ubisemiquinone formed during CoQ reduction by succinate dehydrogenase. Only in situations when reduced flavoprotein in succinate dehydrogenase has no accessible electron transfer partner, higher portion of ubisemiquinone may react under specific conditions
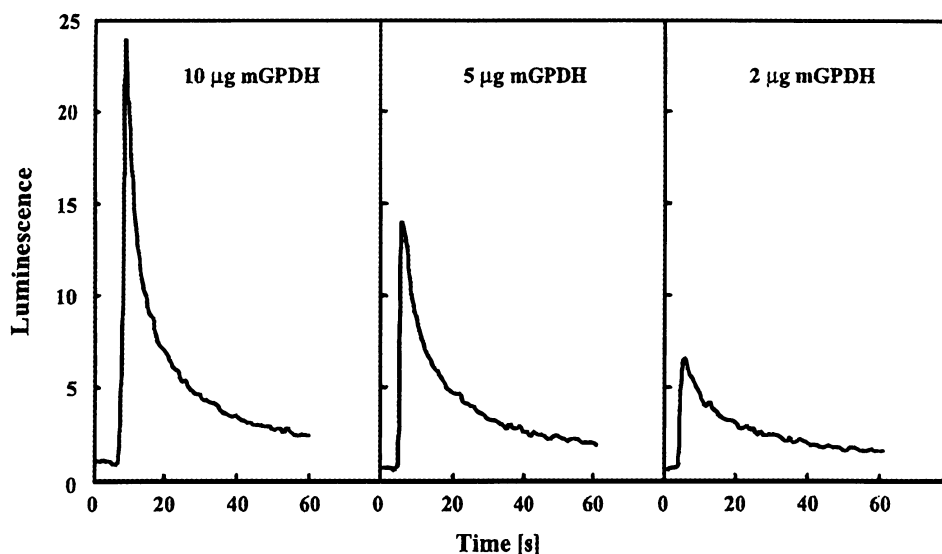
Fig. 7. Glycerophosphate-dependent ferricyanide-induced hydrogen peroxide formation by isolated mGPDH. Luminescence was measured as described in Methods. A total of 2–10 μg protein of isolated enzyme and 10 mM glycerophosphate were used.

with oxygen (Zhang *et al.*, 1998). Because of the absence of CoQ-binding protein, ubisemiquinone formed by mGPDH may be more accessible to oxygen. This should be even more pronounced when the mGPDH reacts with one-electron acceptor ferricyanide (see Fig. 8). Similar activation of ROS production by ferricyanide was also found for isolated succinate dehydrogenase (Zhang *et al.*, 1998).

Absence of the protective effect of CoQ-binding protein thus could explain both the higher ROS production induced by GP than by succinate in the presence of antimycin A (Figs. 1 and 2) and the higher activatory effect of ferricyanide (Figs. 3 and 6). The inhibitory effect of CoQ (Fig. 5) is also fully in agreement with this proposed mechanism (Fig. 8). Increased exogenous concentration of CoQ may evidently substitute the protective effect of CoQ-binding protein. However we cannot exclude that the hydrogen peroxide is formed directly by the interaction of molecular oxygen with reduced flavoprotein moiety of the mGPDH similarly as proposed for the acetyl CoA dehydrogenase (Osumi and Hashimoto, 1978).

Further studies are required to clarify the significance of mGPDH as a ROS producer. It should be clarified whether high activity of mGPDH in BAT, brain, muscle, or pancreas, represents a potential risk, which must be controlled by various defense mechanisms, or whether the peroxide production by mGPDH may have some regulatory significance. For example, in BAT it could participate in activation of apoptotic process, which starts in BAT

when the thermogenic function of BAT is no more required (Lindquist and Rehnmark, 1998) and mitochondria maintain high membrane potential (low respiratory rate) because of absence of hormonal thermogenic stimuli.
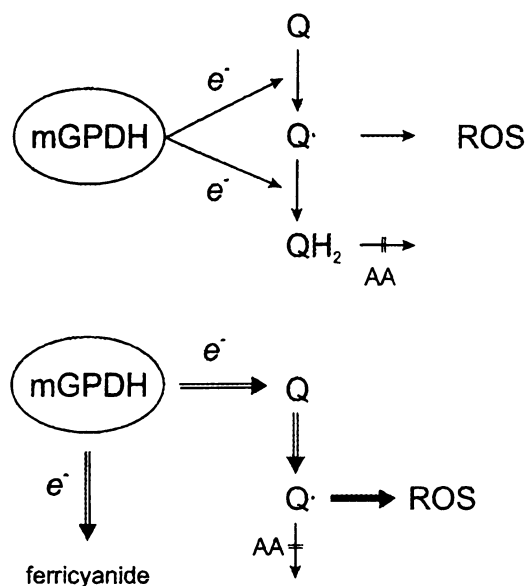

Fig. 8. Proposed mechanism of the activatory effect of potassium ferricyanide on glycerophosphate induced ROS production.

ACKNOWLEDGMENTS

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Article 2

Time-course of hormonal induction of mitochondrial glycerophosphate dehydrogenase biogenesis in rat liver

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Abstract

Thyroid hormones are important regulators of mitochondrial metabolism. Due to their complex mechanism of action, the timescale of different responses varies from minutes to days. In this work, we studied selective T_3 induction of the inner mitochondrial membrane enzyme–glycerophosphate dehydrogenase (mGPDH) in liver of euthyroid rats. We correlated the kinetics of the T_3 level in blood, the mRNA level in liver, the activity and amount of mGPDH in liver mitochondria after a single dose of T_3. The T_3 level reached maximum after 1 h (80 nmol/l) and subsequently rapidly decreased. mGPDH mRNA increased also relatively fast, reaching a maximum after 12 h and fell to the control level after 72 h. An increase of mGPDH activity could be already found after 6 h and reached a maximum after 24 h in accordance with the increase in mGPDH content (2.4-fold vs. 2.7-fold induction). After 72 h, the mGPDH activity showed a significant 30% decrease. When the rats received three subsequent doses of T_3, the increase of mGPDH activity was 2-fold higher than after a single T_3 dose. The results demonstrate that mGPDH displays rapid induction as well as decay upon disappearance of a hormonal stimulus, indicating a rather short half-life of this inner mitochondrial membrane enzyme.
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Keywords: Liver; Mitochondrial GPDH; Triiodothyronine; Mitochondrial protein turnover

1. Introduction

Thyroid hormones (TH) play an important role in the control of cellular metabolism and energy expenditure [1,2]. The mechanism of their action is very complex and involves both a genomic [3,4] and a non-genomic component [5]. The genomic component is represented by thyroid receptors (TR), various coactivators and also transcription factors induced via TR. The so-called non-genomic component is then represented by direct action of TH without effect on nuclear gene transcription, such as changes in intracellular

Ca^{2+} concentration, modulation in activities of several kinases [6] or mitochondrial cytochrome c oxidase (COX) [7]. The timescale in which we can follow the effect of TH might therefore be very different. Furthermore, even the number of TH-controlled genes remains unknown as well as their relative contribution to the regulation of cell energy metabolism.

To better understand these mechanisms, it is advantageous to follow simple metabolic models of TH action. This type of model may be represented by thyroid activation of mitochondrial glycerophosphate dehydrogenase (mGPDH) activity, the effect first described by Lee et al. [8]. In rats fed by a diet containing desiccated thyroid gland, they found a 20-fold increase in mGPDH activity in liver and a 6-fold increase in kidney while no change in mGPDH activity could be detected in heart, skeletal muscle, brain, lung, spleen, adipose tissue and testis [8,9]. Later on, it was

shown that stimulation of gene transcription [10,11] and the subsequent increase in mGPDH protein content [12,13] are responsible for induction of mGPDH enzyme activity. However, there are no sufficient data evaluating the kinetics of this process in its complexity, especially the first phase of hormonal activation and changes during the period when the triiodothyronine (T_3) stimulus peaks off and concentration of serum T_3 is again normalized.

mGPDH represents a rate-limiting component of the glycerophosphate shuttle, an important metabolic pathway connecting glycolysis with the mitochondrial respiratory chain [14,15]. Its up-regulation prevents accumulation of reducing equivalents in the cytosol, derived from T_3-stimulated glycolysis. This useful regulatory pathway is only activated in several tissues or under specific physiological conditions [16]. The high mGPDH expression is also connected with some pathological states such as hyperthyroidism [17] or cancer [18].

In most mammalian tissues, the expression of mGPDH is physiologically highly depressed [19]. However, the reason for this remains unclear. Interestingly, mGPDH might also represent a risk factor for the cell as it is a highly active site of mitochondrial ROS production [20].

Assuming that the TH level and saturation of thyroid receptors in a standard euthyroid rat are in steady-state that is required for the control of cell metabolic functions [21], the model of mGPDH induction in liver might help to clarify the mechanisms underlying the changes in cell metabolic functions in hyperthyroid state. The key importance of mGPDH in the glycerophosphate shuttle allows us to speculate about further consequences of the hyperthyroid state on cell metabolism. Using this model, we can follow the kinetics of enzyme activation in a specific tissue that represents an ideal system to study both the de novo synthesis and protein turnover of the inner mitochondrial energy-converting enzyme that responds rapidly to TH.

The aim of our study was to correlate the level of T_3 in blood, the amount of mRNA coding for mGPDH, the activity of mGPDH and the amount of the mGPDH protein in liver mitochondria after a single dose of T_3 given to euthyroid rats as well as to follow the kinetics of these parameters.

2. Materials and methods

2.1. Animals

Male Wistar rats (Bio-Test Konarovice, Czech Republic) with an initial body weight of 180–220 g were used for the experiments. Animals were housed at 23 ± 1 °C with a 12 h: 12 h light/dark period. The animals had free access to standard laboratory rat chow and water. T_3 at a concentration 20 μg per 100 g of body weight (0.5 ml of solution/100 g body weight) was applied intraperitoneally. Control rats were injected an equal amount of saline solution (0.15 M NaCl). The rats received either one dose of T_3 or three doses in

24-h intervals. The animals were sacrificed after respective time intervals following the last dose of T_3.

2.2. Isolation of mitochondria and enzyme activity measurement

Liver mitochondria were isolated by differential centrifugation in 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA, pH 7.4 [22]. Mitochondria from interscapular brown fat of newborn rats were isolated as described by Hittelman et al. [23]. Isolated mitochondria were stored at −80 °C and frozen–thawed mitochondria were used for measurements.

Activities of mGPDH and succinate dehydrogenase (SDH) were measured using 2,6-dichlorophenol indophenol (DCPIP) as electron acceptor in a medium containing 50 mM KCl, 10 mM Tris–HCl, 1 mM EDTA, 1 mM KCN, pH 7.4. The reaction was started by 20 mM glycerophosphate or succinate and changes of absorbance at 610 nm were subsequently followed. The enzyme activity was calculated using $\varepsilon = 19.1$ cm^2 mol^{-1} as extinction coefficient.

NADH oxidase was measured as oxygen uptake in a medium containing 100 mM KCl, 10 mM Tris–HCl, 3 mM MgCl$_2$, 4 mM K-phosphate, 1 mM EDTA, 2 μM rotenone, 0.4 mM NADH, pH 7.4.

Activity of citrate synthase (CS) [24] and cytochrome c oxidase [25] was measured as in our previous work.

2.3. Serum T_3 concentration measurement

Total and free T_3 in rat blood serum were measured by competitive chemiluminescent enzyme immunoassay using commercial kits "IMMULITE 2000 Total T_3" and "IMMU-LITE 2000 Free T_3" and analyzer DPC IMMULITE 2000 (DPC, USA).

2.4. RNA isolation and Northern blot analysis

Total RNA was extracted using the RNA Blue kit (TOP Bio, Czech Republic). It was then separated on an agarose gel (1.25%) containing 20 mM MOPS (pH 7.0), 6.7% formaldehyde, 50 mM NaAc, and 10 mM EDTA. RNA was transferred to a Hybond-N membrane (Amersham Biosciences, UK) by capillary blotting overnight. The membrane was prehybridized at 42 °C for 2 h in 10 ml/membrane of prehybridizing solution (5× SSC (pH 7.0), 5× Denhardt's, 0.5% SDS, 50 mM sodium phosphate (pH 6.5), 50% formamide, and 100 μg/ml herring sperm DNA) and subsequently hybridized in the same solution with the addition of [^{32}P]dATP-labeled cDNA, labeled by random priming with DECAprime II kit (Ambion, USA).

Blots were hybridized to DNA probe corresponding to the 396-bp fragment of rat mGPDH (bases 306–701 of the ORF). For normalization, a full-length probe corresponding to murine 18S rRNA [26] was used. The radioactive signal was exposed to Phosphor Storage Screen and quantified using the BAS-5000 system (Fuji, Japan). Blots were

stripped and rehybridized sequentially as required in each case.

2.5. Western blot analysis

Tricine-SDS electrophoresis [27] of the samples was performed on 10% polyacrylamide slab gels (Mini Protean II, Bio-Rad, USA) using the same protein aliquots of SDS-solubilised mitochondria (5–10 μg/slot). Proteins from the gel were blotted onto nitrocellulose membrane (Hybond™ C-Extra, Amersham Bioscience, UK) by semi-dry electro-transfer at 0.7 mA/cm² for 1 h.

Incubation conditions and antibodies used for immuno-detection of cytochrome c oxidase, complex I, ATP synthase and SDH were the same as in our previous work [28]. For detection of Core I protein of Complex III we used anti-Core I antibody (1:1000; Molecular Probes A21362) diluted in PBS (0.15 M NaCl, 0.02 M Na_2HPO_4, pH 7.4) with 0.2% Tween 20 and 2% bovine serum albumin. For detection of mGPDH the membranes were blocked overnight with 3% defatted milk and 0.3% Triton X-100 in 0.15 M NaCl and 0.02 M Na_2HPO_4 and incubated for 3 h with rabbit polyclonal anti-mGPDH antibody (1:5000 dilution) raised against the C-terminal peptide LDRRVPIPVDRSCGG of mouse enzyme according to Ueda et al. [29]. Then, the membranes were incubated for 1.5 h using either goat anti-mouse IgG (1:1000; A8924 Sigma, USA) or goat anti-rabbit IgG (1:1000; F0382 Sigma, USA) secondary antibodies conjugated with horseradish peroxidase. The chemilumi-nescent reaction using the ECL kit (Amersham Biosciences, UK) was detected on a LAS 1000 (Fujifilm, Japan) and the signal was quantified using Aida 3.21 Image Analyser software (Raytest, Germany).

Mitochondrial proteins were determined according to Lowry et al. [30].

3. Results

In the initial experiments we used the protocol of T_3 application recommended for induction of mGPDH in liver in previous studies [21,31,32]. Rats received three doses of 20 μg T_3 per 100 g of body weight in 24-h intervals and were

sacrificed 24 or 72 h after the last T_3 application. As demonstrated in Table 1, the concentration of total and free T_3 in serum was four times higher 24 h after the last hormone application. After 72 h, the T_3 concentration returned to control values.

Table 1 also summarizes changes in activities of mGPDH and SDH in isolated liver mitochondria, 24 h after the last T_3 application. The activity of mGPDH was increased 4 times and approximately the same increase was found also 72 h after the last hormone application, when the plasma T_3 already returned back to normal level. In contrast, the increase of SDH activity was only marginal (1.27 and 1.28 times 24 or 72 h after the last T_3 application, respectively) although statistically significant, too (Table 1); 24 h after the last T_3 application, no changes were found in the activities of COX (with or without lauryl malto-side), NADH oxidase (rotenone-sensitive) or CS (not shown).

At the protein level, we also observed a selective increase of mGPDH using specific antibodies against mGPDH, Complex I, Complex II, Complex III, COX and ATP synthase. As demonstrated in Fig. 1B, the increase in mGPDH protein in mitochondria from T_3-treated rats correlates with the increase of the enzyme activity. The content of SDH showed a slight increase (1.1 or 1.3 times after 1 or 3 T_3 injections, respectively), again in accordance with spectrophotometric data. Levels of the representative subunits of other respiratory chain complexes remained unchanged (Fig. 1B and quantification in C). These data thus confirm the specific induction of mGPDH by thyroid hormone in rat liver mitochondria.

To further analyze the kinetics of mGPDH activation, we used a single dose of T_3 and we investigated the time course of changes of T_3 in blood, the amount of mRNA coding for mGPDH in liver and the activity of mGPDH in isolated liver mitochondria. All these data are summarized in Fig. 2.

Immediately after hormone application the level of T_3 in serum highly increased (from 1 to 80 nmol/l). The subsequent decrease was also very rapid with the concentration falling to 15.75 nmol/l after 12 h. Between 12 and 24 h, the concentration of T_3 in blood further decreased to values about 4-fold higher than in control animals (4.25 nmol/l). This kinetics of T_3 decrease is very similar to the

Table 1

Concentration of total and free T_3 in serum and activities of mGPDH and SDH in rat liver mitochondria 24 and 72 h after application of three doses of hormone

	Total T_3 (nmol/l)	Free T_3 (nmol/l)	mGPDH (nmol/min/mg protein)	SDH (nmol/min/mg protein)
Control (A)	1.22±0.24	0.0052±0.0006	4.99±2.25	15.12±4.62
+T_3—24 h (B)	4.92±2.36	0.0246±0.0106	18.28±8.52	19.16±4.81
+T_3—72 h (C)	1.23±0.24	0.0047±0.0014	17.53±5.53	19.29±1.66
(B/A)	4.03 ($P<0.001$)	4.73 ($P<0.001$)	3.66 ($P<0.001$)	1.27 ($P<0.05$)
(C/A)	1.01 (n.s.)	0.90 (n.s.)	3.51 ($P<0.001$)	1.28 ($P<0.01$)

Triiodothyronine was applied 3 times in 24 h intervals and rats were killed 24 and 72 h after the last hormone application. The presented data indicate activities of mGPDH and SDH expressed as nmoles of DCPIP reduced per min per mg protein and average values of total and free triiodothyronine concentration in serum from 3 rats, ± S.E.M.
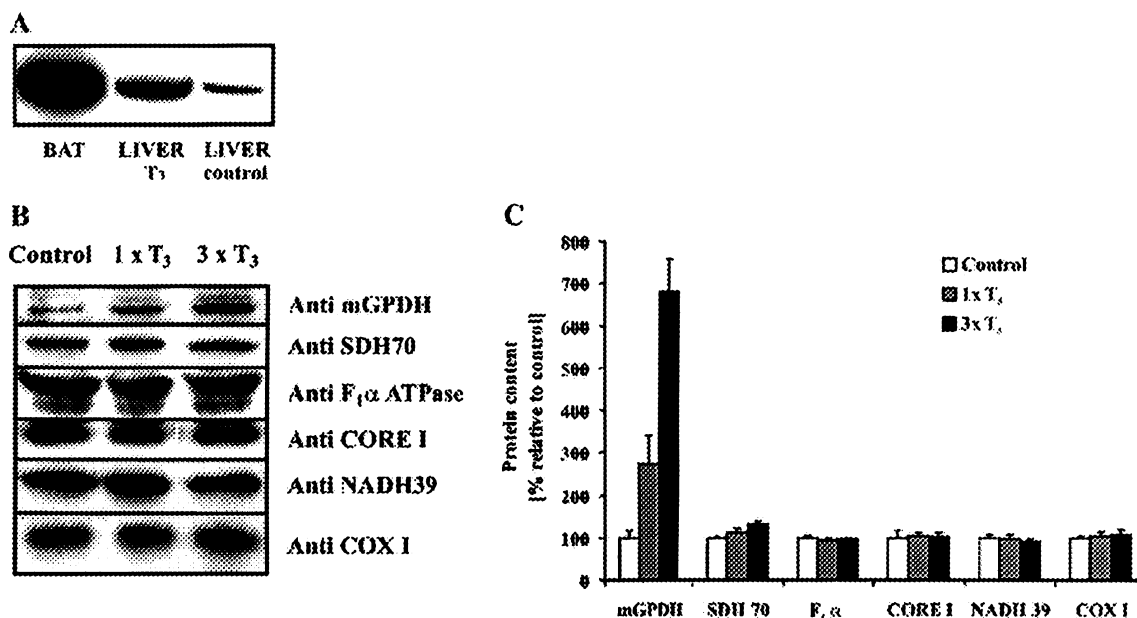
Fig. 1. Detection of mGPDH and respiratory chain enzyme complexes by specific antibodies in control and T_3 treated rats. (A) Mitochondria isolated from brown fat or liver of control rats and liver of rats stimulated with 3 doses of T_3 were analyzed by SDS/PAGE and WB with polyclonal mGPDH antibody. (B) Rats received either 1 dose ($1 \times T_3$) or 3 doses in 24 h intervals ($3 \times T_3$). Liver mitochondria were isolated 24 h after the last dose of T_3 and 10 μg protein aliquots were analyzed by SDS/PAGE and WB. Detection was performed with polyclonal antibody against mGPDH and monoclonal antibodies against SDH (subunit SDH70), ATPase (subunit F_1), Complex III (Core I protein), Complex I (subunit NADH39) and COX (subunit COX I). Representative Western blot is shown. (C) Quantification of data from 3 to 5 Western blots performed as in panel B.

situation in the previous experiment, where three doses of T_3 had been used (compare with Table 1).

The increase of mGPDH transcript is faster (with significant increase found already 3 h after T_3 had been applied) than the increase of mGPDH enzyme activity in isolated mitochondria. From Fig. 2, it is also evident that the activation of mRNA synthesis is highly dependent on the
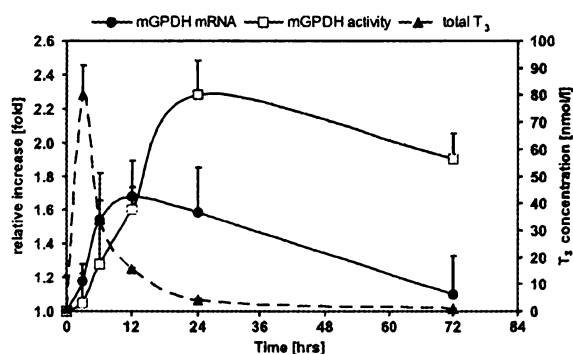

Fig. 2. Correlation of changes of T_3 in blood, mGPDH mRNA in liver and mGPDH activity in liver mitochondria after a single dose of hormone. Rats received a single dose of T_3 and were sacrificed at the respective time following the injection. Blood was collected for total T_3 measurement and liver for total RNA and mitochondria isolation. The levels of total T_3 in serum ( ) are presented in nmol/l, those of the mGPDH transcript (normalized to 18 S rRNA) (●) and mGPDH protein activities ( ) are presented as relative increase versus control values. Representative trace for one experimental series is shown.

concentration of T_3 in blood. The amount of mRNA in liver begins to decline 24 h after T_3 application, which correlates well with the decline of T_3 concentration in blood. However, when the T_3 level in blood returns to control values, a significant 20% increase of mRNA content can still be detected.

At the protein level a significant increase of mGPDH activity in isolated mitochondria could be detected 6 h after the hormone application while the maximum values were reached after 24 h. In comparison with rats obtaining 3 doses of T_3 (Table 1) the increase of the enzyme activity was about 50% lower. Due to the semiquantitative nature of Western blot, we followed the kinetics as changes in the enzyme activity. Nevertheless, the increase of protein content after 24 h (Fig. 1B and C) correlates well with the increase of the enzyme activity (2.4-fold induction when measured as enzyme activity vs. 2.7-fold induction in protein content). This can exclude the possible kinetic activation of mGPDH and indicates that de novo synthesis of the enzyme is responsible for induction of mGPDH biogenesis. The enzyme activity fell by 30% between 24 and 72 h after T_3 application, evidently due to reduced mRNA synthesis.

4. Discussion

In this work, we attempted to define the interrelationship between hormone dose, its plasma concentration and

induction of mGPDH in rat liver as a function of time. We have clearly demonstrated that mGPDH is the only component of mammalian respiratory chain that is rapidly synthesized upon T_3 stimulus and displays fast turnover, which are characteristics essential for rapid "on demand" regulation according to different cellular needs.

Expression of mGPDH is highly depressed in most mammalian tissues [19]. A high mGPDH content is found only in brown fat [16] and much lower, but still significant activities are found in placenta [33], testes, pancreatic beta-cells [13] and cultured fibroblasts [24]. An important regulatory role in mGPDH biogenesis is played by thyroid and steroid hormones. Detailed mechanism of their action is still not well understood due to the complexity of this regulation.

Various aspects of T_3-dependent mGPDH induction have already been extensively studied [19,21,34–36]. Nevertheless, to our knowledge, this is the first study to match the T_3 concentration in serum, mRNA and protein content as well as the enzyme activity of mGPDH.

We confirmed previous findings that indicated the specific action of thyroid hormones on mGPDH. Using specific antibodies, we could also find proportional increases in mGPDH protein content both after one or three doses of T_3. In further experiments, we used the model of mGPDH biogenesis induction after a single dose of T_3 to euthyroid rats. Due to the simplicity of this system, we could follow kinetics of enzyme biogenesis, which cannot be analyzed in the traditionally used system of repeated doses of T_3.

Although the induction of mGPDH enzyme activity after a single dose of T_3 has already been studied by Oppenheimer et al. [21], they measured only the kinetics of enzyme activity changes. Furthermore, to ensure full occupancy of thyroid receptors they used a high dose of T_3 (200 or 5000 µg/100 g body weight). Enzyme activity began to rise 13 h after injection and it grew until >95% of TR was occupied with T_3. They also studied the decay of enzyme activity and calculated the mGPDH half-life to be 2.8 days. These data generally correspond to our findings. However, contrary to Oppenheimer, we used one to two orders of magnitude lower concentrations of T_3 (20 µg/100 g body weight), which might better resemble the situation in hyperthyroidism. Accordingly, the extent of mGPDH induction is also lower in our experiments. From the comparison of our data after single or three doses, it is evident that we do not work at TR-saturating concentrations.

We found that the mGPDH transcript is induced 3 h after T_3 injection. This is similar to the data of Dummler, where the increase appeared after 4–6 h [37]. The mGPDH transcript is therefore the earliest of all nucleus-encoded mitochondrial proteins induced by T_3 and its speed more resembles cytosolic T_3-regulated proteins, such as malic enzyme [38].

Transcription of the mGPDH gene is activated by TR/RXR binding to the thyroid hormone response element (TRE) sequence in the promotor region of the gene [39,40]. The temporal expression pattern of mGPDH mRNA indicates that it belongs to the family of T_3 fast-response transcripts induced directly by liganded TR. Another mode of T_3 action is through induction of transcription and subsequent synthesis of other transcription factors or coactivators involved in mitochondrial biogenesis (e.g. NRF-1 or PGC-1) [6]. Genes regulated in this manner respond to T_3 after a much longer period of time (48 h) necessary for all intermediate processes to take place [6,41]. Early induction of the mGPDH transcript therefore speaks against the involvement of such a pathway in the regulation of mGPDH biosynthesis.

Another important aspect of the present study is evaluation of mGPDH protein turnover. So far, there are very few studies dealing with the turnover of mitochondrial proteins [42–44]. In fact, detailed analysis of the half-life of mitochondrial OXPHOS enzymes and other proteins from different mitochondrial compartments as well as data on tissue specificity are still missing. Some studies implicated macroautophagy to be responsible for mitochondria degradation [45,46]. However, quantitative studies in liver cells indicated that macroautophagy might be responsible for degradation of no more than a half of the mitochondrial proteins [46]. Furthermore, studies on liver, cultured hepatocytes or hepatoma cells indicated that the half-life of mitochondrial proteins is very variable with $t_{1/2}$ ranging from 1.2 to 185 h in the case of matrix proteins, and from 35 to 156 h for membrane proteins [43,47,48]. This reflects a selective degradation of proteins that are no longer needed for mitochondrial function, presumably by members of the AAA-protease family [49]. From our enzyme activity measurements, we may estimate the mGPDH half-life to be approximately 60 h. This relatively fast turnover indicates the potential existence of a selective degradation pathway that may be operating to ensure faster mGPDH decay, when compared with other proteins of the inner mitochondrial membrane.

Thyroid hormone activation of mGPDH in liver represents a useful model system for studies of mechanisms participating in hormonal regulation of biogenesis of mitochondrial enzymes. This model system can also help to better understand the role of mGPDH and the glycerophosphate shuttle in the regulation of cell metabolic processes.

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Article 3

Specific Properties of Heavy Fraction of Mitochondria from Human-term Placenta – Glycerophosphate-dependent Hydrogen Peroxide Production

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Mitochondrial respiratory chain enzyme Complexes are present in placenta at proportion similar to other tissues with exception of glycerophosphate dehydrogenase (mGPDH) which is expressed at a very high rate. As shown by Western blot quantification and respiratory chain enzyme activity measurements, the specific content of mGPDH is similar to that of succinate dehydrogenase or NADH dehydrogenase. Using fluorometric probe dichlorodihydrofluorescein diacetate we found that placental mitochondria display high rate of glycerophosphate-dependent hydrogen peroxide production. This was confirmed by oxygraphic detection of glycerophosphate-induced, KCN- or antimycin A-insensitive oxygen uptake. Hydrogen peroxide production by mGPDH was highly activated by one-electron acceptor, potassium ferricyanide and it was depressed by inhibitors of mGPDH and by cytochrome c. Our results indicate that mGPDH should be considered as an additional source of reactive oxygen species participating in induction of oxidative stress in placenta.

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Keywords: Human placenta; Mitochondria; Glycerophosphate dehydrogenase; ROS

Abbreviations: COX, cytochrome c oxidase; H2DCFDA, dichlorodihydrofluorescein diacetate; GCCR, glycerophosphate-cytochrome c oxidoreductase; NCCR, NADH-cytochrome c oxidoreductase; SCCR, succinate-cytochrome c oxidoreductase; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; ROS, reactive oxygen species

INTRODUCTION

Placenta is a transient organ acting as both physical and functional interface between mother and developing fetus. Placenta and the fetal membrane provide fetus with the nutrition required for its growth and it serves also as an excretory organ eliminating waste products [1]. Other important functions of placenta are fetal protection, gas exchange [2] and synthesis of a wide range of proteins and hormones [3,4]. To fulfill all these functions, placenta is metabolically very active organ in which energy metabolism has an important role and placental defects of energy provision system may have serious impact on fetal developmental processes.

Mitochondria play an important role in energy provision in placenta that depends mainly on glucose utilization [5–7]. We

focused our attention on factors that could negatively affect mitochondrial oxidative phosphorylation process in placenta and thus alter growth process during fetal development. Placental mitochondria provide important biological model for basic studies of human mitochondria [8] and they are of key importance for understanding the process of energy provision for placental growth and fetus maturation during intrauterine development [1,9,10]. Placental mitochondria are involved in energy metabolism disturbances of very premature neonates and they participate in generation of reactive oxygen species (ROS) during placental development and in pathologic pregnancies, such as those related to preeclampsia [11–13].

Placenta is an organ with heterogeneous cell populations and dynamic transformation during the fetal development [14]. This makes studies of mitochondrial energy provision system very complicated. Early studies used mitochondria prepared from whole placenta [15]. The oxidative capacity of mitochondria was found to be very low with variable values of coupling. Only when the methods for isolation of

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mitochondria were improved, higher rates of respiration and higher respiratory control ratios were obtained [16—18]. Nevertheless, many findings remained unexplained: low content of cytochromes and adenine nucleotides [19], low cytochrome c oxidase (COX) activity, low respiratory rate of NADH-dependent substrates [17,19], presence of specific phosphatases bound to placental mitochondria [19—21] as well as localization of metabolically and structurally different mitochondrial populations [17]. Also a paradox of relatively high capacity of mitochondrial respiratory chain enzymes and findings indicating that most of energy derived from glucose comes from glycolysis [1,22] needs further elucidation.

Part of these problems was solved by studies of Gellerich et al. [19], Martinez et al. [23] and Matsubara et al. [14]. They characterized two mitochondrial types: bigger mitochondria from trophoblasts (light fraction), and smaller and more dense mitochondria from syncytiotrophoblasts (heavy fraction). The latter had higher activity of succinate dehydrogenase, citrate synthase and very high activity of cytochrome P_{450}, which participates in steroidogenesis.

Another specific property found in placental mitochondria was unusually high activity of mitochondrial glycerophosphate dehydrogenase (mGPDH) [18,24,25], when compared to most other mammalian tissues. The physiological significance of the high activity of mGPDH is not clear. This enzyme is essential for operation of glycerophosphate shuttle [26], an important regulatory device of intermediary metabolism, but on the other hand it represents potent site of ROS production in mitochondria [27,28].

Therefore we analyzed the mGPDH content in placental mitochondria with respect to other respiratory chain enzymes and tried to demonstrate to what extent the high mGPDH activity correlates with glycerophosphate-dependent hydrogen peroxide production, because this could indicate a potential risk of the high mGPDH activity for increased ROS production in placenta.

MATERIALS AND METHODS

Isolation of mitochondria from human-term placenta

Mitochondria were isolated from human-term placenta according to Martinez et al. [23]. For better separation of heavy and light mitochondria the pH of the resolving medium was increased to 7.8 [17]. Placental samples were dissected and washed several times in washing medium (0.25 M sucrose, 0.154 M KCl, 1 mM EDTA, pH 7.4). Washed placenta was homogenized at 0 °C in the isolation medium (0.25 M sucrose, 10 mM Tris—HCl, 1 mM EDTA, pH 7.4) using a glass—Teflon homogenizer. A 15—20% homogenate was centrifuged at 4 °C for 10 min at 750 g. Supernatant was filtered through gauze and centrifuged at 4 °C for 10 min at 10800 g. The mitochondrial pellet was suspended in resolving medium (0.25 M sucrose, 1 mM EDTA, 1 mM Tris—HCl, pH 7.8) and centrifuged for 10 min at 12000 g. The pellet

obtained consists of two phases, heavy and light fraction of mitochondria. Both phases were carefully separated, suspended in the isolation medium and centrifuged at 4 °C for 10 min at 10800 g. After the last washing, heavy and light fractions were suspended in isolation medium and used for analysis.

Isolation of rat liver, hamster brown fat and muscle mitochondria

Liver mitochondria from adult Wistar rats were isolated in 0.25 M sucrose, 10 mM Tris—HCl, 1 mM EDTA, pH 7.4 according to Schneider and Hogeboom [29]. Brown fat mitochondria were isolated in the same sucrose medium according to Hittelman et al. [30], from 2-month-old Syrian hamsters, cold adapted at 4 °C for 3 weeks. Muscle mitochondria were isolated according to Makinen and Lee [31], but without use of protease.

Detection of mitochondrial enzymes with specific antibodies

The samples of human-term placenta, hamster brown fat, rat liver and muscle mitochondria for SDS-PAGE were boiled for 3 min in a sample-lysis buffer (100 mM Tris—HCl, pH 7.0, 2% (v/v) mercaptoethanol, 4% (w/v) SDS, 10% (v/v) glycerol). SDS—Tricine electrophoresis [32] was performed on 10% polyacrylamide slab gels (Mini protean, BioRad) using the same protein aliquots of SDS-solubilized mitochondria (5—10 µg/slot). Proteins from the gel were blotted onto nitrocellulose membrane (Hybond™ C EXTRA, Amersham Bioscience) by semi-dry electrotransfer at 0.3—0.7 mA/cm² for 1 h.

For immunodetection of respiratory chain enzymes, the membranes were blocked with PBS (0.15 M NaCl, 0.02 M Na_2HPO_4, pH 7.4) plus 0.2% Tween 20 (PBST) and incubated for 2.5 h with the primary monoclonal antibodies against subunits of Complexes I, II, III, IV and V, respectively, diluted in PBST containing 2% bovine serum albumin: anti-NADH39 subunit of Complex I (1:250 dilution; Molecular Probes A-11140), anti-70 kDa subunit of Complex II (1:2000; Molecular Probes A-11142), anti-Core1 protein of Complex III (1:1000; Molecular Probes A21362), anti-COXVIc subunit of Complex IV (1:200; Molecular Probes A-6401), anti-ATPase $F_1—\alpha$ subunit of Complex V (1:200000; lot 20D6 [33]). For detection of mGPDH, the membranes were blocked over night with 3% defatted milk and 0.3% Triton X-100 in 0.15 M NaCl and 0.02 M Na_2HPO_4 and incubated for 3 h with rabbit polyclonal anti-mGPDH antibody (1:5000 dilution) raised against C-terminal peptide LDRRVPIPVDRSCGG of mouse enzyme according to Ueda et al. [34]. Then the membranes were incubated for 1.5 h using either goat anti-mouse IgG (1:1000; A8924 Sigma) or goat anti-rabbit IgG (1:1000; F0382 Sigma) secondary antibodies conjugated with horseradish peroxidase. The chemiluminescent reaction using ECL kit (Amersham Biosciences) was detected on a LAS 1000 (Fujifilm) and signal

Table 2. Oxygen uptake of human-term placental mitochondria with different substrates

Additions	Oxygen uptake (pmol oxygen/s/mg protein)
GP	109.4 ± 9.2
GP + cyto	169.6 ± 5.8
GP + cyto + ADP	292.3 ± 6.1
GP + cyto + ADP + FCCP	310.5 ± 19.6
GP + cyto + ADP + FCCP + KCN (RCI = 1.72 ± 0.13)	22.3 ± 5.2
SUC	110.4 ± 7.3
SUC + cyto	140.7 ± 17.5
SUC + cyto + ADP	260.0 ± 21.0
SUC + cyto + ADP + FCCP	307.0 ± 23.0
SUC + cyto + ADP + FCCP + KCN (RCI = 1.84 ± 0.15)	15.9 ± 5.2
GLU + MAL + cyto	61.2 ± 10.4
GLU + MAL + cyto + ADP	152.1 ± 17.0
GLU + MAL + cyto + ADP + ROT (RCI = 2.49 ± 0.13)	24.4 ± 6.1
PYR + MAL + cyto	66.1 ± 7.0
PYR + MAL + cyto + ADP	130.1 ± 17.0
PYR + MAL + cyto + ADP + ROT (RCI = 1.97 ± 0.23)	19.6 ± 3.5
ASC + TMPD + cyto	725.5 ± 45.6
ASC + TMPD + cyto + KCN	2.3 ± 3.2

Freshly isolated human-term placental mitochondria were incubated in 100 mM KCl, 10 mM Tris-HCl, 4 mM K-phosphate, 3 mM MgCl₂, 1mM EDTA, 1 mM EGTA, 0.6 mg/ml bovine serum albumin fatty acid free, pH 7.3 at 30 °C. Where indicated 10 mM glycerophosphate (GP). 10 mM succinate (SUC), 10 mM glutamate (GLU), 10 mM pyruvate (PYR), 3 mM malate (MAL), 20 µM cytochrome c (cyto), 1 mM ADP, 3µM rotenone (ROT), 5 mM ascorbate (ASC), 1 mM tetramethyl-p-phenylenediamine (TMPD) were added. Protein was 0.5 mg/ml. RCI indicates respiratory control index for particular substrate. Measurements with glycerophosphate and succinate were repeated five times and measurements with glutamate, malate and ascorbate were repeated three times with different mitochondrial preparations from human-term placentas. Data indicate means ± SEM.

approximately 50% of COX activity in the presence of ascorbate and TMPD was insensitive to KCN, in our placental mitochondria COX activity was almost completely inhibited by KCN (Table 2).

Determination of NCCR showed that the rotenone-insensitive NCCR activity was almost four-fold higher than GCCR or SCCR (Table 1). However, the rotenone-sensitive fraction was quite comparable to the activity of GCCR or SCCR. High rotenone-insensitive activity is evidently connected with a high microsomal contamination as observed also in previous studies [19,42] or with high activity of NCCR of outer mitochondrial membrane, which is also rotenone insensitive [43].

These measurements of Complex I activity differed from previously published oxygraphic data [17,19] that showed very low respiration of placental mitochondria with NADH-dependent substrates in comparison with succinate- or

glycerophosphate-dependent respiration. Also in our oxygraphic studies we found the rate of glutamate and malate or pyruvate and malate respiration to be only about 50% of that of succinate- or glycerophosphate-dependent respiration (Table 2). This discrepancy between spectrophotometric and oxygraphic measurements could indicate some limitations in transport of NADH-dependent substrates to placental mitochondria or modified function of NADH oxidase. To further investigate this we measured rotenone-sensitive NADH oxidation by oxygraphy. In frozen-thawed placental mitochondria we found the rate of NADH oxidation (Table 3) to be three to four times lower than the rate of glycerophosphate or succinate oxidation. The lower rate of NADH oxidation was apparently not due to limited permeability of frozen mitochondria for NADH, because the same results were obtained with frozen-thawed and sonicated mitochondria (Table 3). Oxidation of all respiratory substrates tested on frozen-thawed and sonicated mitochondria was not activated by uncoupler and was almost completely inhibited by KCN (Table 3).

These results suggest lower content or down-regulated activity of Complex I in placental mitochondria. We therefore determined specific content of individual respiratory chain Complexes by Western blot analysis using subunit-specific monoclonal antibodies against Complexes I, II, III, IV and V

Table 3. NADH, glycerophosphate and succinate oxidation by frozen-thawed placental mitochondria

Additions	Oxygen uptake (pmol oxygen/s/mg protein)
NADH	85.4
NADH + ROT	32.4
NADH + ROT + cyto	270.7
NADH + ROT + cyto + KCN	3.5
NADH	84.6
NADH + cyto	360.8
NADH + cyto + FCCP	340.0
NADH + cyto + FCCP + ROT	263.3
NADH + cyto + FCCP + ROT + KCN	2.5
*NADH	82.6
*NADH + cyto	208.1
*NADH + cyto + FCCP	216.0
*NADH + cyto + FCCP + ROT	163.9
*NADH + cyto + FCCP + ROT + KCN	4.7
GP	75.2
GP + cyto	425.3
GP + cyto + KCN	2.9
SUC	74.5
SUC + cyto	322.2
SUC + cyto + KCN	4.7

Where indicated (*) aliquot of frozen-thawed mitochondria was sonicated three times for 15 s at 0 °C. Incubation conditions were the same as in Table 2. NADH was 0.4 mM, glycerophoshate (GP) 10 mM, succinate (SUC) 10 mM, cytochrome c (cyto) 40 µM, rotenone (ROT) 3 µM. Mitochondrial protein was 0.4 mg/ml.

(CI, CII, CIII, CIV and CV). As demonstrated in Figure 1A, the relative proportion between individual Complexes was quite similar in human placenta, mouse liver and mouse muscle mitochondria. The respective ratios of antigen signals CI:CII:CIII:CIV:CV normalized to CIV signal were found to be 0.8:0.6:0.9:1:1 in placenta, 0.7:0.7:0.6:1:1 in liver and 0.7:0.3:0.6:1:0.8 in muscle. Apparently, the content of CI in placenta was quite comparable with that in other tissues.

Furthermore, we used the same approach to quantify the content of mGPDH. In this case we used highly specific rabbit antibody raised against C-terminal peptide of human mGPDH. As apparent from Figure 1B, placental mitochondria contained about two-fold higher amount of mGPDH than liver mitochondria which corresponded to about 38% of the mGPDH content in brown adipose tissue mitochondria. These amounts correlated well with enzyme activities of GCCR in the respective types of mitochondria (brown adipose tissue (BAT) 720 $\pm$ 50.3 nmol/min/mg protein (n = 5), liver 21 $\pm$ 7.7 nmol/min/mg protein (n = 4), placenta 107 $\pm$ 1.2 nmol/min/mg protein (n = 3)).

Glycerophosphate-dependent hydrogen peroxide production in placental mitochondria

In our previous studies [27,28] we found that mGPDH represents a new site of ROS production in the mitochondrial respiratory chain. Using fluorometric detection of hydrogen peroxide generation by H_2DCFDA we could demonstrate a high rate of glycerophosphate-dependent hydrogen peroxide production also in placental mitochondria. Under the same experimental conditions, succinate- or NADH-dependent hydrogen peroxide production was several times less intensive (Figure 2).

Glycerophosphate-dependent hydrogen peroxide production can also be measured as a KCN-insensitive oxygen uptake. Similarly as in brown fat [27] and liver mitochondria [44], also in placental mitochondria we found glycerophosphate-dependent hydrogen peroxide production that was about seven-fold increased by one-electron acceptor, potassium ferricyanide (Figure 3, Table 4). In spectrophotometric measurements performed in parallel, we followed reduction of added ferricyanide. The decline of ferricyanide-induced oxygen uptake correlated with complete reduction of added ferricyanide (not shown). As shown in Figure 4, when the added ferricyanide was reduced by mGPDH, the rate of hydrogen peroxide production returned again to the original values. This activation of oxygen uptake could be repeated several times by addition of another portion of oxidized ferricyanide (Figure 4). From oxygen uptake curves both the total oxygen consumption connected with the ferricyanide reduction and the ratio
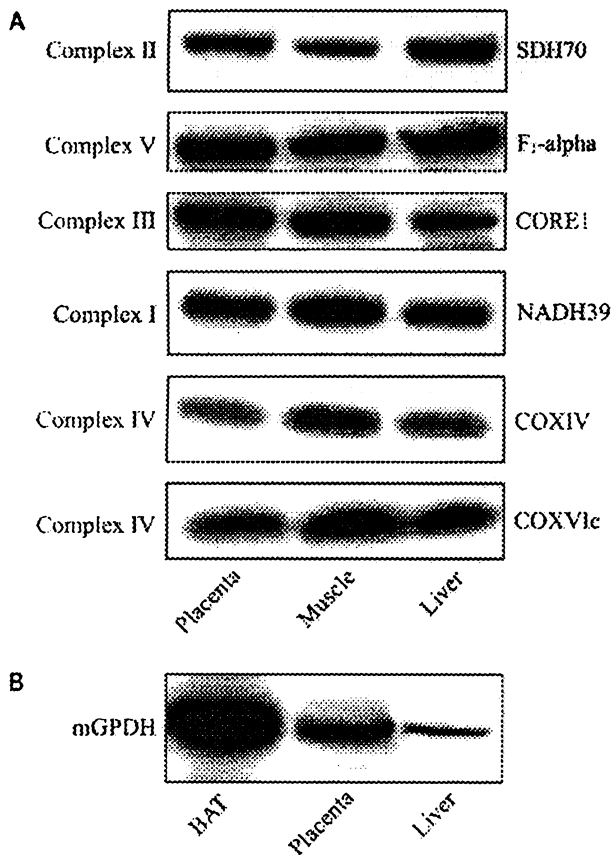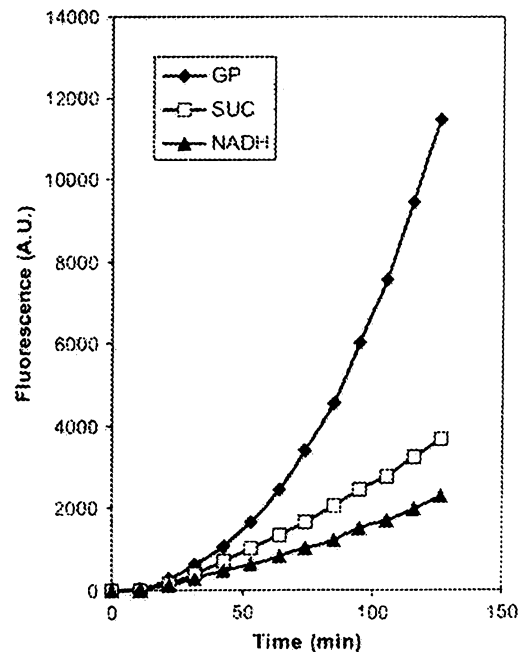
Figure 1. Immunodetection of the respiratory chain enzymes by specific antibodies. In (A), SDS-polyacrylamide gel electrophoresis and Western blot analysis was performed in isolated mitochondria from human placenta, rat muscle and rat liver using monoclonal antibodies against subunits of respiratory chain Complex I (NADH39), Complex II (SDH70), Complex III (COREI), Complex IV (COXIV and COXVIc) and Complex V (F1-alpha). In (B), the same analysis was performed in isolated mitochondria from hamster brown adipose tissue (BAT), human placenta and rat liver mitochondria using polyclonal antibody against mGPDH.

Figure 2. Fluorometric detection of hydrogen peroxide generation by placental mitochondria. Frozen—thawed mitochondria (0.05 mg protein/ml) were incubated in 1 ml of 100 mM KCl, 20 mM Tris—HCl, 4 mM K-phosphate, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4 in the presence of 2 µM H_2DCFDA and 2 µM antimycin A. Ten millimoles of glycerophosphate (GP), 10 mM succinate (SUC) or 0.4 mM NADH was used as a substrate.
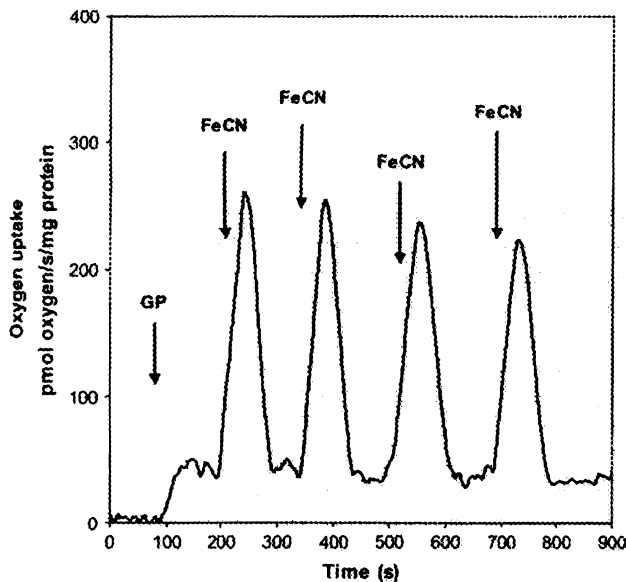
Figure 3. Ferricyanide-activated, glycerophosphate-dependent hydrogen peroxide generation by human placental mitochondria. Freshly isolated mitochondria (1 mg protein/ml) were incubated at 30 °C in 100 mM KCl, 20 mM Tris–HCl, 4 mM K-phosphate, 3 mM MgCl₂, 1 mM EDTA, pH 7.4 and 1 mM KCN. Ten millimoles of glycerophosphate (GP) and 63 μM potassium ferricyanide (FeCN), were added as indicated. The experiment was repeated twice using two different placental mitochondria preparations with the same results.

between ngAt O consumed per nmol of ferricyanide added can be calculated (Table 4). This value indicates efficiency of the glycerophosphate-dependent hydrogen peroxide generation. Values about 0.3 ngAt O per nmol potassium ferricyanide are comparable to those obtained in our previous experiments with brown fat mitochondria [27,28]. Contrary to glycerophosphate, succinate was not able to support hydrogen peroxide generation under the same experimental conditions (Figure 4). In this experiment we used antimycin A as the respiratory chain inhibitor and we obtained the same results as in previous experiments with KCN (Figure 3).

Fifty percent inhibition of mGPDH activity by 50 μM mercaptodicarbanonaborate [41] led to decline in hydrogen peroxide production rate (Figure 5), however, the total amount of hydrogen peroxide produced with respect to added ferricyanide remained unchanged. The inhibition of mGPDH activity by mercaptodicarbanonaborate can be completely eliminated by bovine serum albumin [41]. Similarly, addition of serum albumin after mercaptodicarbanonaborate inhibition completely recovered the ferricyanide-induced, glycerophosphate-dependent hydrogen peroxide production (Figure 5), which confirms direct involvement of mGPDH in this reaction.

Cytochrome c is known as a potent endogenous scavenger of electrons [45]. Figure 5 demonstrates that increasing concentration of cytochrome c strongly depressed glycerophosphate-dependent hydrogen peroxide production.

DISCUSSION

In this work we focused on detailed characterization of mitochondria isolated from human-term placenta, in order to clarify discrepancies in published data on activities of various mitochondrial enzymes and their proportions. For our studies we used heavy mitochondrial fraction that displayed approximately two-fold higher activity of all measured respiratory chain enzymes than the light mitochondrial fraction. Activities of rotenone-sensitive NCCR, SCCR and GCCR were quite similar; however, when measured as oxygen uptake, we found (in accordance with others e.g. [19]) a lower rate of oxygen uptake with NADH-dependent substrates than with succinate or glycerophosphate. Using specific antibodies we found that the amount of respiratory chain Complex I is not reduced in placental mitochondria and the proportions among Complexes I, II, III, IV and V are similar to those in other mammalian tissues. From our experiments and from other published data (e.g. [19]) we may conclude that mitochondria in placenta are capable of regular ATP production by coupled oxidative phosphorylation and have normal content of respiratory chain

Table 4. Glycerophosphate-dependent, ferricyanide-induced hydrogen peroxide generation by mitochondria from human-term placenta

	Rate of oxygen uptake (pmol/s/mg protein)		Total oxygen uptake (ngAt O)		O/FeCN (ngAt O/nmol FeCN)	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Mitochondria						
+KCN	2.0	3.5	–	–	–	–
+10 mM GP	44.6	50.2	–	–	–	–
+FeCN	256.6	270.3	22.09	21.45	0.35	0.34
+FeCN	251.2	265.4	20.66	20.35	0.33	0.32
+FeCN	234.7	248.7	22.58	22.13	0.36	0.35
+FeCN	221.9	232.5	20.21	21.08	0.32	0.33

Data in Table 4 were calculated from the oxygraphic trace presented in Figure 3. Freshly isolated placental mitochondria were incubated in 100 mM KCl, 20 mM Tris–HCl, 4 mM K-phosphate, 3 mM MgCl₂, 1 mM EDTA, pH 7.4 at 30 °C. Mitochondrial protein was 1 mg/ml and KCN was 1 mM. Sequential additions of 63 nmol/ml of potassium ferricyanide (FeCN) are shown in Figure 3. The experiment was repeated twice with the same results using two different mitochondrial preparations from human placentas.
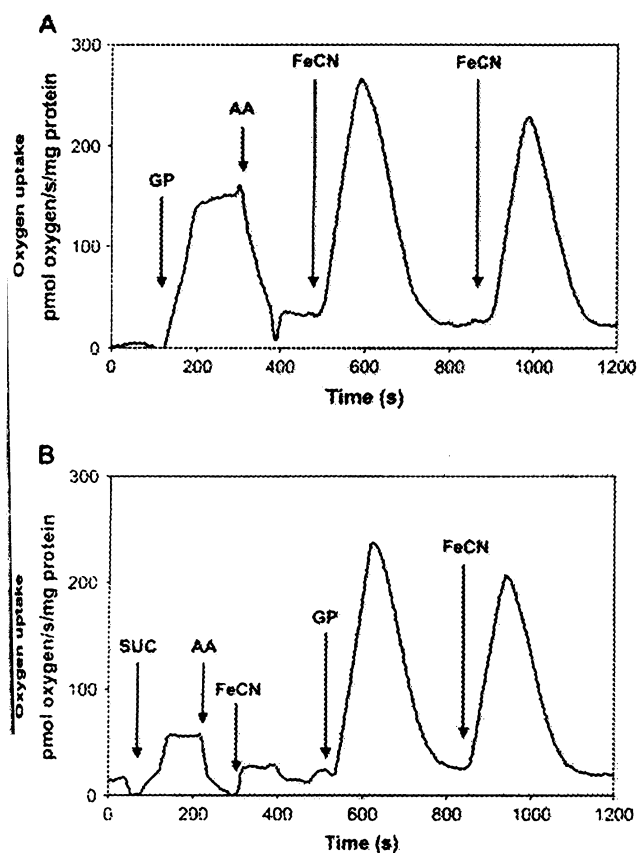
A

B

Figure 4. Ferricyanide-induced hydrogen peroxide production in the presence of glycerophosphate (A) and succinate (B). Frozen–thawed placental mitochondria were incubated at 30 °C in 100 mM KCl, 20 mM Tris–HCl, 5 mM K-phosphate, 3 mM MgCl₂, 1 mM EDTA, pH 7.4. In (A), where indicated, 10 mM glycerophosphate (GP), 1 µM antimycin A (AA), and twice 5 µM potassium ferricyanide (FeCN), were added. In (B), where indicated, 5 mM succinate (SUC), 1 µM antimycin A (AA), 10 mM glycerophosphate (GP) and 63 µM potassium ferricyanide (FeCN), were added. Mitochondrial protein was 0.46 mg/ml.

ymes. Taken together, the immunodetection of respiratory Complexes is in good agreement with spectrophotometric activity measurements. Activity of all measured cytochrome reductases exceeds the activity of respective oxidases and the most pronounced difference shows Complex I. It appears that activity of NADH oxidase is somehow down-regulated in placental mitochondria, because the oxidation of both NADH-dependent substrates in intact mitochondria and NADH in frozen–thawed and sonicated mitochondria is lower than that of glycerophosphate and succinate. However, further experiments will be required to clarify this discrepancy between NADH-cytochrome c oxidoreductase activity and oxygraphic measurements where the Complex I activity is measured in the context of the whole respiratory chain.

Placental mitochondria possess many specific structural and metabolic properties when compared to mitochondria from other tissues. They have very high activity of nonspecific ATPase [21] and alkaline phosphatase [19] that split adenine nucleotides and diminish the ADP/O ratio. They have also very high activity of rotenone-insensitive NADH oxidase.
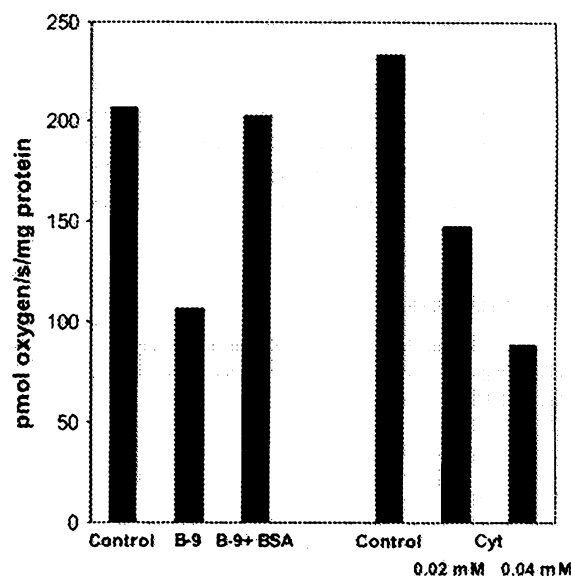
Control B-9 B-9+BSA Control Cyt

0.02 mM   0.04 mM

Figure 5. Inhibition of glycerophosphate-induced hydrogen peroxide production by mercaptodicarbanonaborate and cytochrome c. Incubation conditions were the same as in Figure 4. Mitochondrial protein was 0.5 mg/ml. KCN-insensitive oxygen uptake was induced by addition of 10 mM glycerophosphate (GP) and 63 µM potassium ferricyanide (FeCN). Glycerophosphate-dependent, FeCN-activated hydrogen peroxide production was measured in the presence of 50 µM mercaptodicarbanonaborate (B-9), or B-9 and 1 mg/ml bovine serum albumin fatty acid free (B-9 + BSA). Inhibition of cytochrome c, was measured in the presence of 20 µM or 40 µM cytochrome c (Cyt). The experiment was repeated twice using two different placental mitochondria preparations with the same results.

Indeed, the high activity of rotenone-insensitive NADH dehydrogenase can be the result of contamination by terminal mixed function NADH/NADPH oxidases [46] and/or by rotenone-insensitive NADH-cytochrome c oxidase localized on the outer mitochondrial membrane. It also remains unclear whether these specific metabolic properties of isolated mitochondria are connected with all, or only some cellular types present in placenta.

Very specific feature of placental mitochondria is unusually high activity of mGPDH. High activity of this enzyme enables function of the glycerophosphate shuttle [24] that is a very useful regulatory device which can reoxidize cytosolic NADH and maintain the high rate of glycolysis without production of lactic acid. For placenta this pathway is especially important because in placenta glycolysis represents a very important pathway in cell energy metabolism [1,5–7]. In most mammalian tissues the expression of mGPDH is highly depressed, placental cells thus could represent a useful model system for elucidation of mechanisms controlling mGPDH biogenesis in human tissues.

Recently we found that mGPDH beside its positive role in activation of glycolysis, represents also a potential risk for mammalian cells as a generator of ROS [27,28]. Our data presented in this work show that a significant glycerophosphate-dependent hydrogen peroxide production can be detected also in placental mitochondria. Glycerophosphate-dependent hydrogen peroxide generation in combination with a very high

activity of NAD(P)H-dependent superoxide production [12] and high activity of NO synthase [12,13] could participate in disturbances of placental development as well as in placental pathologic processes connected with oxidative stress [11,12]. Glycerophosphate-dependent ROS production might be important also in diabetic hyperglycemia [22,47] since the activated NADH production might increase in placental

mitochondria the electron flow from mGPDH. Further studies, however, will be necessary to evaluate to what extent glycerophosphate-dependent ROS generation can be considered as potential risk due to its participation in acceleration of pathological alterations in placenta and to what extent it could play an important role as a signaling mechanism in metabolic regulations as was recently proposed [12,48,49].

ACKNOWLEDGMENTS

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Article 4

Review

Mitochondrial diseases and ATPase defects of nuclear origin

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Abstract

Dysfunctions of the F_1F_o-ATPase complex cause severe mitochondrial diseases affecting primarily the paediatric population. While in the maternally inherited ATPase defects due to mtDNA mutations in the *ATP6* gene the enzyme is structurally and functionally modified, in ATPase defects of nuclear origin mitochondria contain a decreased amount of otherwise normal enzyme. In this case biosynthesis of ATPase is down-regulated due to a block at the early stage of enzyme assembly—formation of the F_1 catalytic part. The pathogenetic mechanism implicates dysfunction of Atp12 or other F_1-specific assembly factors. For cellular energetics, however, the negative consequences may be quite similar irrespective of whether the ATPase dysfunction is of mitochondrial or nuclear origin.
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Keywords: Mitochondrial disease; Cardiomyopathy; ATP synthase; Oxidative phosphorylation; Respiratory chain complex

1. Introduction

Last decade of bioenergetic research on mitochondrial diseases has uncovered an increasing number of human mitochondrial disorders that are caused by mutations in nuclear genes encoding the subunits of oxidative phosphorylation complexes (OXPHOS), or other proteins that are essential either for the biosynthesis of specific cofactors (such as heme) or assembly of heterooligomeric OXPHOS complexes from individual subunits (for review, see Ref. [1]). Up to now, numerous mutations in nuclear genes have been shown to cause a dysfunction of all mitochondrial respiratory chain complexes, NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), bc_1 complex (Complex III), cytochrome c oxidase (Complex IV) and also F_1F_o-ATP synthase (ATPase, Complex V). Very often, these defects manifest rather early and affect paediatric population. Interestingly, the Complex I defects are mostly connected with mutations in genes encoding the subunits

of the complex, while Complex IV and Complex III defects are caused by mutations in specific assembly proteins or biosynthetic factors. To the same category apparently belong the ATPase deficiencies of nuclear origin that are rather rare and biochemically manifest as a reduction of cellular content of ATPase complex that is otherwise structurally and functionally normal.

2. Selective defects of mitochondrial ATPase

Mitochondrial ATPase is the key enzyme of cellular energy conversion. ATPase uses the H^+ gradient generated by the respiratory chain as a driving force for the synthesis of ATP from ADP and phosphate. The mammalian ATPase complex is formed by 16 different subunits [2] and consists of the globular F_1 catalytic part connected by two stalks to the membrane-embedded F_o moiety, which translocates protons across the inner mitochondrial membrane. Two F_o subunits, subunit a (ATP6) and A6L (ATP8), are coded for by mitochondrial DNA (mtDNA) [3]; all other subunits are nuclearly encoded. Mitochondrial encephalomyopathies due to selective defects in mitochondrial ATPase are less frequent than the disorders of the respiratory chain complexes. They are mostly very severe and can be caused by

mtDNA mutations as well as by mutations in nuclear genes.

3. ATP6 mutations

All maternally inherited primary ATPase defects are associated with subunit a (see Ref. [4]), an essential component, together with multiple copies of the subunit of the ATPase proton channel [5,6]. The ATP6 gene is a hotspot of pathogenic mtDNA mutations affecting ATPase; no mutations have been found in the ATP8 gene. ATP6 mutations are mostly missense heteroplasmic mtDNA mutations affecting the protonophoric function of the subunit a. Higher prevalence show T8993G(C) mutations [7,8] which change Leu[156] to Arg or Pro. At a lower mutation load they are manifested as a NARP syndrome (Neurogenic muscle weakness, Ataxia, Retinitis Pigmentosa), at heteroplasmy exceeding 90% they present as maternally inherited Leigh syndrome (severe and fatal encephalopathy). Less frequent are T9176G(C) mutations which change Leu[217] [9,10] or a T8851G mutation [11] affecting Trp[109], both manifesting also as striatal necrosis syndromes (see Ref. [4]). Impairment of the ATPase H^{+} channel by different ATP6 mutations results often, but not always, in decreased ATP production, while the ATPase hydrolytic activity remains unchanged [12,13]. Mitochondria from T8993G cells are capable of ATP-dependent proton translocation [14] indicating that at least the vectorial proton transport by the enzyme from matrix to cytosol is unaffected. Increased lability of the ATPase complex, possibly due to altered assembly of subunit a, apparent as accumulation of incomplete ATPase assemblies, has been described in T8993G mutation [13,15]. Interestingly, these assembly intermediates could not be found in some other cases [16], which indicates that, similarly as in segregation of ATP6 mutations [17], a different nuclear background and participation of putative regulatory factor(s) may be involved in their pathogenetic mechanism.

A completely different type of pathogenic mechanism is represented by homoplasmic mtDNA 2-bp microdeletion 9205delTA, so far found in two cases only [18,19]. This mutation removes the stop codon of the ATP6 gene and affects the cleavage site between ATP6 and COXIII transcripts. The biochemical and clinical presentations of these two cases are, however, strikingly different [20–22]. An involvement of some nuclear-encoded factor operating at the level of mitochondrial RNA processing is to be expected. Several proteins have been described in yeast (NCA2, NCA3, NAM1/MTF2, Aep3p) that are essential for proper processing of mitochondrial RNAs, namely of the ATP8-ATP6 cotranscript (see Ref. [23]), but their mammalian orthologues were not found, possibly reflecting differences in the structure of mammalian mitochondrial RNAs that, in comparison with yeast, lack introns and 3- and 5-prime untranslated regions. Another group of factors involved in translation of mitochondrial RNAs is represented by proteins mediating mRNA-ribosome interactions. Search for mammalian orthologues in this group was more successful and an LRPPRC protein was identified using functional genomics approach [24]. It was shown that mutation in the LRPPRC gene causes the Leigh syndrome of French-Canadian type, which is a human mitochondrial COX deficiency [25]. Identification of additional mammalian factors specific for other mitochondrial transcripts can be foreseen.

4. ATPase defects of nuclear origin

ATPase defects due to a nuclear genome mutations where an alteration of mtDNA genes was excluded are characterised as selective decrease of ATPase content that is caused by diminished biosynthesis of the ATPase complex. An increasing number of cases diagnosed recently (Table 1) indicates that these defects may be more frequent than originally expected. ATPase deficiency of possible non-mitochondrial origin was first described in a child with 3-methylglutaconic aciduria and severe lactic acidosis [26]. An extremely low ATPase activity and low, tightly coupled, respiration rates were observed in muscle mitochondria, but no mutation was found in mtDNA genes encoding ATPase subunits. The nuclear origin of ATP synthase deficiency was demonstrated for the first time in 1999 [27] in a new type of fatal mitochondrial disorder. A child with severe lactic acidosis, cardiomyopathy and hepatomegaly died 2 days

Table 1
Patients with mitochondrial ATPase deficiency

| Case	ATPase (%)	Onset/ survival	Biochemical data and phenotype	Reference
I	<10	neonate/15 months	LA, methylglutaconic aciduria, CM, developmental delay	[26]
IIa	<30	neonate/2 days	fatal LA, CM, hepatomegaly	[27]
IIb	<30	neonate/2 days	fatal LA, CM	
IIIa	<20	neonate/32 days	LA, CM, respiratory failure	[22]
IIIb	<30	neonate/3 years	LA, CM, short statute, failure to thrive, developmental delay	
IV	<30	neonate/8 years	LA, methylglutaconic aciduria, CM, developmental delay	[22]
V	<10	neonate/3 years	LA, CM, developmental delay	[28]
VI	<20	neonate/14 months	LA, methylglutaconic aciduria, dysmorphism, enlarged liver, marked brain atrophy	[29]
VII	<30	neonate/3 days	fatal LA, enlarged liver, small left ventricle, intracranial haemorrhage	[29]

LA—lactic acidosis, CM—cardiomyopathy.
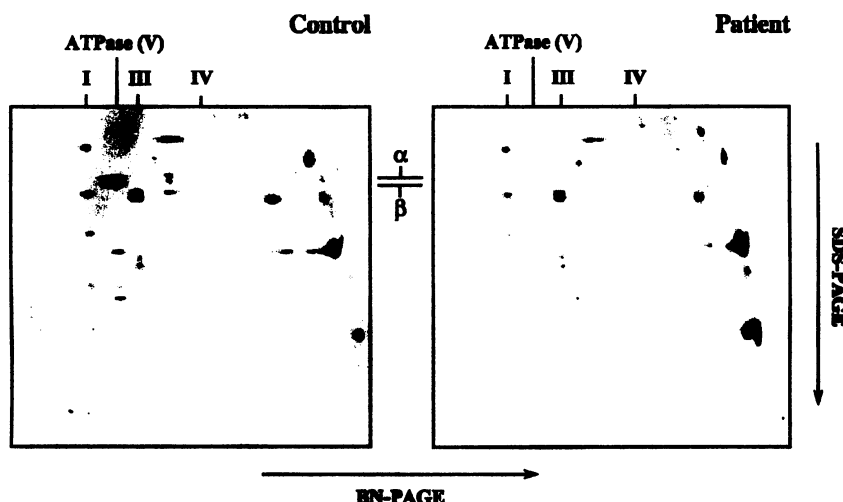
Fig. 1. OXPHOS complexes in a patient with ATPase deficiency. Blue-Native/SDS two-dimensional electrophoresis was performed using laurylmaltoside-solubilised proteins from heart mitochondria from patient (IIa in   ) and control, the gel was stained with Coomassie blue. The position of ATPase complex and of respiratory chain complexes I, III and IV is indicated; white arrows point to α/β F_1-ATPase subunits.

after birth. A generalised, 70–80% decrease in ATPase activity and ATP production was associated with corresponding selective decrease of the content of the ATPase complex, which had normal size and subunit composition   . Transmitochondrial cybrid cells made of patient's fibroblasts fully complemented the ATPase defect and confirmed the nuclear origin of impaired biogenesis of the enzyme complex   . Later on, two other similar cases of selective ATPase deficiency were found in the Czech Republic   and one in Austria   . Most recently, two patients with selective ATPase deficiency were described in Belgium   . In one of them, De Meirleir et al. located the pathogenic mutation in the *ATP12* gene for the first time (see further). As summarised in   , most of the cases showed a reduction of ATPase content to <30% of the control, early onset of the disease, cardiomyopathy and survival of several days or weeks. Interestingly, methylglutaconic aciduria was found in several longer surviving patients. One of them showed a different phenotype of degenerative encephalopathy characterised by cortical and subcortical atrophy   .

5. Altered biosynthesis of ATPase

The mechanism of ATPase biosynthesis is still not very well understood. The mammalian enzyme is expected to assemble stepwise   . Several transient assembly intermediates have been identified with initial formation of the F_1 catalytic part to which the nuclear encoded membrane sector subunits are added, followed by mtDNA-encoded subunits   . Native forms of ATPase can be easily revealed by Blue-Native electrophoresis   that became a very powerful approach to diagnostics of mitochondrial OXPHOS defects today. We have performed detailed stud-

ies on most of the diagnosed cases of selective ATPase deficiency and always found only a full-size ATPase complex to be present in cells and isolated mitochondria from the patient's tissues   . No accumulation of assembly intermediates analogous to subcomplexes observed in *ATP6* mutations or cells with doxycycline-inhibited translation of mitochondrial proteins   could be detected in fibroblasts with ATPase defects of nuclear origin. In [^{35}S]-methionine labelling experiments, decreased biosynthesis of the assembled ATPase has been found   . It contrasted with the increased biosynthesis of the β subunit of the F_1 catalytic part that had a very short half-life   . The cells also showed extramitochondrial accumulation of the β subunit, supporting the view that the biogenesis of ATPase is disturbed at an early stage when the F_1 catalytic part is formed. ·

6. F_1-ATPase assembly and affected nuclear factor(s)

The possible cause of insufficient ATPase biosynthesis can be a limited production or mutation of some of the
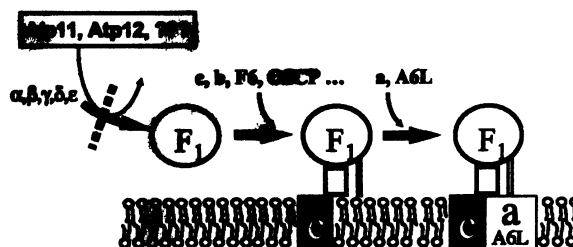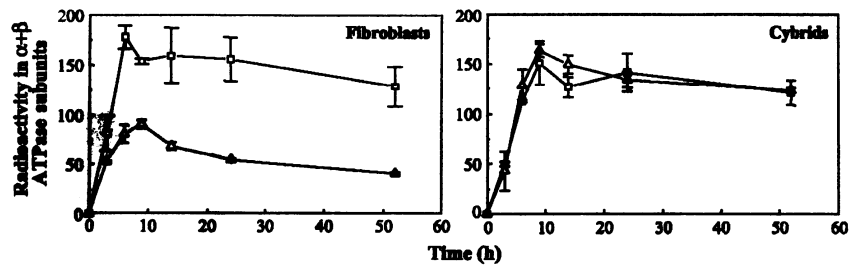

Fig. 2. ATPase assembly scheme.

Fig. 3. Metabolic labelling of ATPase-deficient fibroblasts and derived transmitochondrial cybrids. Cells from patient (△) and control (□) were labelled for 3 h with [^{35}S]methionine, followed by 3–48-h chase with excess cold methionine. Aliquots of solubilised OXPHOS complexes were analysed by two-dimensional electrophoresis and radioactivity was determined by phosphorimaging (patient IIa in   ).

ATPase subunits. The cellular content of ATPase differs in mammalian tissues and it may vary also during ontogenetic development. Physiologically ATPase is down-regulated about 10-fold, relative to other OXPHOS complexes, in thermogenic brown fat where the electrochemical potential of proton gradient is dissipated in the form of heat by the uncoupling protein UCP1   . Interestingly, the two-dimensional electrophoretic pattern of OXPHOS proteins in brown fat is almost identical with that found in patients with ATPase deficiency. In brown fat a selective transcriptional down-regulation of subunit c isogenes P1 and P2 and availability of the subunit c has been found as a limiting step for de novo synthesis of ATPase   . The transcriptional control of subunit c genes has been implicated in control of the cellular content of ATPase also in other mammalian tissues   . However, in ATPase-deficient patients expression of subunit c isogenes was normal or even increased   and the mRNA levels for other ATPase subunits did not show a significant decrease. As also sequencing of cDNAs of F_1 and F_0 subunits from patient cells was unable to detect any pathogenic mutation, the possible cause of the disease might be associated with a dysfunction of an ATPase-specific assembly factor.

Biosynthesis of eukaryotic ATPase is a highly ordered process, which involves several ATPase-specific assembly proteins. Studies in yeast identified five chaperone-type factors necessary for the assembly of the functional enzyme. Atp10p and Atp22p were found to mediate F_0 assembly   while Atp11p, Atp12p and Fmc1p are required for the F_1 part   . Fmc1p is essential at elevated growth temperatures but FMC1 deletion could be rescued by overexpression of Atp12p   . Recently, human orthologues of Atp11p and Atp12p (Atpaf1p and Atpaf2p according to new nomenclature) have also been identified. Like the yeast proteins, human Atp11p has chaperone-like activity toward the β subunit and human Atp12p interacts with the α subunit   . Expression of ATP11 and ATP12 in mammalian tissues is about two orders of magnitude lower than expression of genes for the corresponding F_1 subunits, in accordance with the chaperone function of these proteins. Interestingly, ATP11 expression in mouse is nearly constant in all tissues, indicating that ATP11 rather behaves like a maintenance

gene. In contrast, ATP12 expression differed up to 30-fold in diferent tissues and it was found to correlate well with mRNA levels of both F_1-α and F_1-β (BAT >> kidney, liver > heart, brain > skeletal muscle), showing the highest mRNA level in the thermogenic, ATPase-poor brown adipose tissue

.

The most recent molecular genetic studies by De Meirleir et al.   in two patients from Belgium with ATPase deficiency identified in one case the first mutation in ATP12 (patient 6 in   ), which was homozygous TGG-AGG missense mutation in exon 3 changing Trp^{94} to Arg. No mutation in ATP11 and ATP12 was present in the second case. Similarly, we were unable to find any mutation in other cases that have been analysed (patients II–V in   ). Moreover, we were also unable to detect any decrease in ATP11 and ATP12 transcripts in these patients that would indicate their decreased content. Taken together, the available data suggest an involvement of another, yet unidentified ATPase assembly factor. This view might also be supported by the fact that the phenotype of the patient with ATP12 mutation—marked brain atrophy, significantly differs from other patients presenting with cardiomyopathy   .

7. Biochemical changes in nuclear ATPase defects

Pronounced reduction of ATPase content in patient's fibroblasts strongly decreased the synthesis of ATP, ADP-stimulated respiration, and the discharge of mitochondrial membrane potential which showed even higher steady state values than the control cells [22,27,28]. However, even in cells with 90% decrease of ATPase content, a significant ATP production could be seen [28]. As apparent from inhibitor titration studies [45,46], individual OXPHOS enzymes of mitochondrial energy metabolism can be inhibited to a certain extent without noticeable reduction of the mitochondrial coupled respiration rate. These threshold effects are different for individual OXPHOS complexes and they also display tissue specificity. In the case of ATPase, some 10% of normal activity of the enzyme was found to be sufficient for 30–60% functionality of the whole respiratory chain, depending on the type of tissue. This would mean that, with respect to mitochondrial energy

provision, a significant decrease of ATPase capacity can be tolerated, at least under conditions when the energetic demands of the patient's organism are rather low.

For the pathogenetic mechanism of ATPase deficiency thus may be more relevant the mitochondrial ROS production related to the higher levels of $\Delta\Psi_m$, observed in

patient's fibroblasts. An exponential increase of mitochondrial ROS production occurs at high levels of $\Delta\Psi_m$ above 140 mV   . On the contrary, decrease of $\Delta\Psi_m$ via stimulation of ATP synthase activity, a low ATP/ADP ratio, and substrate limitation or increased proton permeability due to external or internal uncoupling lower the amount of
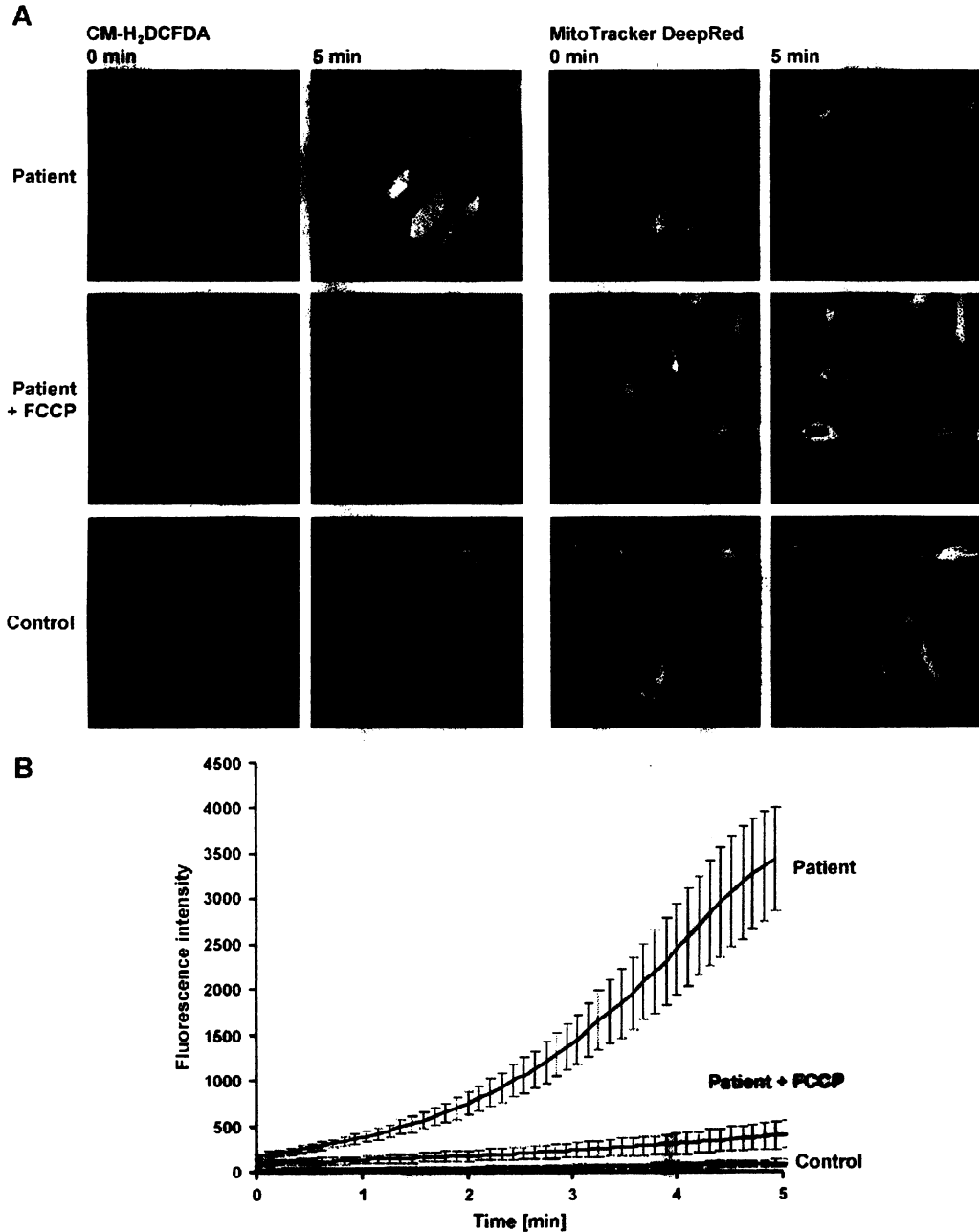

Fig. 4. ROS production analysed by confocal microscopy in ATPase-deficient cells. Intact fibroblasts (patient IV in   ) were labelled with 1 µM CM-H₂DCFDA (5-(and-6)-chloromethyl-2′ ,7′ -dichlorodihydrofluorescein diacetate) and 50 nM Mitotracker DeepRed and analysed on a Leica TCS SP2 microscope. (A) Increase in green fluorescence serves as a measure of ROS production in cell, as non-fluorescent CM-H₂DCFDA is oxidized by ROS to fluorescent CM-DCFDA (fluorescein derivative). (B) Quantification of confocal images is shown. To compensate for mitochondrial content in patient and control cells, CM-DCFDA fluorescence was related to Mitotracker fluorescence.

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Article 5

Two components in pathogenic mechanism of mitochondrial ATPase deficiency: Energy deprivation and ROS production

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Abstract

Isolated defects of mitochondrial ATPase due to diminished biosynthesis of the enzyme represent new class of severe mitochondrial diseases of nuclear origin. The primary cause of decreased cellular content of ATPase appears to be a problem in assembly of the F_1 catalytic part of the enzyme. With the aim to elucidate how the low ATPase content affects mitochondrial energy provision and ROS production, we have investigated fibroblasts from patients with ATPase decrease to 10–30%. Measurements of cellular respiration showed pronounced decrease in ATPase capacity for basal respiration, mitochondrial ATP synthesis was decreased to 26–33%. Cytofluorometric analysis using TMRM revealed altered discharge of mitochondrial membrane potential ($\Delta\Psi_m$) in patient cells, which was 20 mV increased at state 3-ADP. Analysis of ROS production by CM-H$_2$DCFDA demonstrated 2-fold increase in ROS production in patient cells compared to controls. ROS production rate was sensitive to uncoupler (FCCP) and thus apparently related to increased $\Delta\Psi_m$. Our studies clearly demonstrate that low ATPase content and decreased mitochondrial ATP production lead to high values of $\Delta\Psi_m$ and are associated with activation of ROS generation by the mitochondrial respiratory chain. In conclusion, both the energetic deprivation and increased oxidative stress are important components of the pathogenic mechanism of ATPase disorders.

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Keywords: Mitochondria; ATPase deficiency; Respiration; Membrane potential; ROS

1. Introduction

Insufficient or altered function of mitochondrial oxidative phosphorylation (OXPHOS) system represents primary cause of human mitochondrial OXPHOS diseases, broad range of pathological states that vary in age of onset, severity and phenotypic presentation (DiMauro, 2004). Underlying genetic defects include mutations both in mitochondrial DNA and in numerous nuclear genes. While the mtDNA mutations frequently affect adult populations, nuclear genetic defects are usually associated with early onset (Shoubridge, 2001). Mitochondrial dysfunction has been also shown to play a role in the pathogenesis of late-onset neurodegenerative disorders such as Parkinson, Alzheimer or Huntington diseases and especially in the most common human disease—process of aging. (Wallace, 1992).

Mitochondrial ATP synthase (ATPase) represents the key enzyme of mitochondrial energetic machinery being responsible for synthesis of most of cellular ATP. ATPase, or complex V of the mitochondrial respiratory chain, phosphorylates intramitochondrial ADP at the expense of proton gradient generated by the respiratory chain complexes I, III and IV. ATPase complex consists of the catalytic F_1 part connected by two stalks with the membrane embedded F_0 part that constitutes the proton channel (Walker and Collinson, 1994). The ATPase holoenzyme is composed of 16 different subunits. Only two of them, the F_0 subunits a (ATP6) and A6L (ATP8), are encoded by the mtDNA (Anderson et al., 1981).

ATPase defects typically present as severe, early onset, mostly fatal diseases. Obviously, understanding of the molecular pathogenic mechanism of ATPase disorders is essential for both the diagnostics and therapy of the disease. There are two types of ATPase defects known today that differ both genetically and phenotypically. The first type is a 'qualitative change' of ATPase complex due to maternally transmitted mtDNA missense mutations in *ATP6* gene, resulting in altered function of ATPase proton channel and striatal necrosis syndromes (Schon et al., 2001). The second type is a 'quantitative deficiency' of ATPase complex, an isolated defect of nuclear origin in biosynthesis of the enzyme that appears to be stalled at an early stage of enzyme assembly

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(Houstek et al., 1999). The ATPase complex is structurally and functionally normal, but its specific content relative to other respiratory chain enzymes decreases to 10–30% of the control. Most of known cases show cardiomyopathy while the brain is not affected (Houstek et al., 2004).

Dysfunction of ATPase can decrease mitochondrial synthesis of ATP with obvious, severe consequences for all energy-dependent cellular functions. In addition, recent studies indicate that both types of ATPase defects are associated with increased oxidative stress due to elevated mitochondrial ROS production (Houstek et al., 2004; Mattiazzi et al., 2004). Nevertheless, it remains completely unclear why the isolated dysfunction of the same enzyme differs so much in the phenotypic presentation. Perhaps, the variable extent of the two components of the pathogenic mechanism, energy deprivation and oxidative stress may be responsible.

In this report, we investigated in detail fibroblasts from four previously described patients (Houstek et al., 2004) with isolated ATPase deficiency of the latter type with the aim to find out how the low ATPase content limits mitochondrial energy provision and to what extent the insufficient discharge of mitochondrial proton gradient affects the ROS production by the mitochondrial respiratory chain.

2. Materials and methods

2.1. Cell cultures

Human skin fibroblast from controls and four patients (Patients IIa, IIIa, IV, V, previously described in Houstek et al. (2004)) with ATPase content decrease to 10–30% were cultured in DMEM medium (SEVAC, Czech Republic) with 10% fetal calf serum (Sigma, USA) at 37 C in 5% CO_2 in air. Cells were grown to approximately 90% confluence and harvested using 0.05% trypsine and 0.02% EDTA. Detached cells were diluted with ice-cold cultured medium, sedimented by centrifugation and washed twice in cold phosphate-buffered saline (PBS). The protein concentration was measured by Bio-Rad protein assay (Germany).

2.2. High-resolution respirometry

Respiration of intact fibroblasts suspended in 2 ml D-MEM supplemented with 4.5 g/l glucose (protein concentration 0.3–0.8 mg/ml) at 30 C was measured using Oxygraph-2k (Oroboros, Austria). A steady-state respiration without any additional substrates was followed for 5 min and then ATPase inhibitor aurovertin was sequentially titrated to final concentrations of 8–1544 nM. The protocol was completed by addition of uncoupler FCCP up to optimum concentration (0.6–1.5 μM) for maximal stimulation of respiratory rate. The data were analyzed with DatLab2 software (Oroboros, Austria), the rates of oxygen consumption were normalized on protein content and expressed as pmol s^{-1} mg^{-1} protein.

2.3. ATP synthesis

The rate of ATP synthesis was measured at 37 C in 150 mM KCl, 25 mM Tris–HCl, 10 mM potassium phosphate, 2 mM EDTA, 1% (w:v) BSA, pH 7.2, using 0.5 mM ADP and 10 mM succinate or 10 mM pyruvate + 10 mM malate as a substrate, as described before (Wanders et al., 1996). Protein concentration was 1 mg/ml. For permeabilisation of fibroblasts 0.1 mg digitonin/mg protein was used (Fluka, USA). The reaction was started by addition of fibroblasts and performed for 15 min. ATP content was determined in DMSO-quenched samples by luciferase assay according to Ouhabi et al. (1998). The ATP production was expressed in nmole ATP/min per mg protein.

2.4. Flow cytometry analysis of mitochondrial membrane potential $\Delta\Psi_m$

The cells were resuspended in 80 mM KCl, 10 mM Tris–HCl, 3 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM EDTA, pH 7.4, 10 mM succinate at a protein concentration 0.2 mg/ml, permeabilized with 0.1 mg digitonin/mg protein (Fluka, USA) and incubated with 20 nM TMRM (Molecular Probes, USA) for 15 min. To determine mitochondrial content, the cells were incubated with 20 nM MitoTracker Green (MTG, Molecular Probes, USA), a specific mitochondrial marker. Cytofluorimetric analysis was performed on the PAS-III flow cytometer (Partec, Germany) equipped with a 488 nm Ar–Kr laser. TMRM fluorescence was analyzed in the FL2 channel (band pass filter 580±30 nm) and MTG fluorescence in the FL1 channel (band pass filter 530±15 nm). Data were acquired in FloMax software (Partec, Germany) and analyzed with Summit Offline V3.1 software (Cytomation, USA). A minimum of 10,000 cells were used and arithmetic mean value of the fluorescence intensity was determined for each sample. Normalized fluorescence intensities were obtained by dividing the TMRM signal by the MTG signal. The changes in $\Delta\Psi_m$ were calculated according to Plasek et al. (2005).

2.5. Fluorometric detection of ROS production

For determination of ROS production in intact cells fluorescent probe 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes, USA) was used. Cells were grown in multi-well culture plates in presence or absence of uncoupler. For measurement, culture medium was changed for 135 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, pH 7.4, with 1 μM CM-H_2DCFDA. The formation of the fluorescent compound, dichlorofluorescin, was monitored with excitation set to 485±7.5 nm and emission to 535±15 nm using a Victor II Multilabel Counter (Wallac, Finland).

2.6. Statistical analysis

All the data were analyzed by the conventional statistical methods using the Student's t test in MS Excel.

3. Results

In order to evaluate the mitochondrial energy metabolism with special focus on function of ATPase in fibroblasts from patients suffering from isolated deficiency of mitochondrial ATPase, we analyzed the respiration in intact fibroblasts respiring on glucose in D-MEM medium used for cell cultivation (routine basal respiration). To assess how the low ATPase content limits the cellular respiration, we measured respiration at conditions when ATPase is inhibited to different extent using titration by aurovertin, a highly specific inhibitor of the catalytic F_1 part of the enzyme. As apparent from Fig. 1, the respiration of patient cells shows much higher sensitivity to inhibition of ATPase resulting in 6-fold decrease in I_{50}. This difference is in agreement with 70–90% decrease of ATPase content determined in patient cells in previous studies (Houstek et al., 1999; Houstek et al., 2004; Mayr et al., 2004) and means that the ATPase threshold for coupled respiration is very low. Thus, the capacity for ATP production at conditions when energy demands are increased is very likely to be insufficient in patient cells.

To test the ability of cells to synthesize ATP by mitochondrial oxidative phosphorylation system, we analyzed ATP production of digitonine-permeabilised cells supplied with different respiratory substrates. Fig. 2 shows that ATP production was very low in patient cells compared with controls using NADH-dependent substrates, pyruvate and malate, or substrate for complex II, succinate. The ATP production in patient cells was decreased to 26 and 33%, respectively. As expected, the decrease was more pronounced at conditions when the electron transport respiration includes complex I and higher P/O ratio values.

Low capacity of ATPase at conditions when respiratory chain is sufficiently supplied with substrates implies that the ability to discharge the proton gradient should be affected in patient cells. In further experiments we determined the mitochondrial membrane potential ($\Delta\Psi_m$) at conditions

Fig. 2. Decreased mitochondrial ATP production in ATPase-deficient patient cells. ATP production was determined in digitonine-permeabilised cells supplied with 0.5 mM ADP and respiratory substrates 10 mM pyruvate+ 10 mM malate or 10 mM succinate in the presence of 1 µM rotenone. Data are expressed in nmol ATP/min per mg protein and represent mean+SD of analysis of four different patient fibroblasts and controls, *p   0.01.

analogous to the measurements of ATP production. Cells were permeabilized by digitonin, supplied with succinate and mitochondrial membrane potential was analyzed by flow cytometry using cationic, $\Delta\Psi_m$- sensitive probe TMRM. The TMRM signal was normalized to the mitochondrial content using MitoTracker Green that specifically and independently on $\Delta\Psi_m$ labels mitochondria and therefore serves as a measure of mitochondrial mass. As shown in Fig. 3, at state 4, when mitochondria are supplied with substrate in the absence of ADP, comparably high values of $\Delta\Psi_m$ were found in patient and control cells. After addition of ADP the TMRM fluorescence decreased to about 23% of state 4 value in control cells but only to 50% in patient cells. Aurovertin fully recovered original state 4 fluorescence in both cell types. These data clearly indicate that patient cells maintain elevated values of $\Delta\Psi_m$ in vivo, when mitochondria are energetically active and produce ATP. Calculation of $\Delta\Psi_m$ changes in
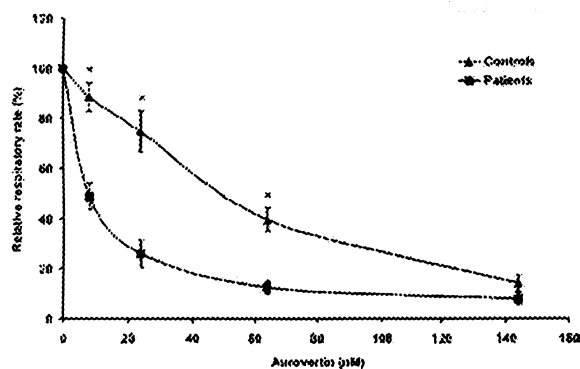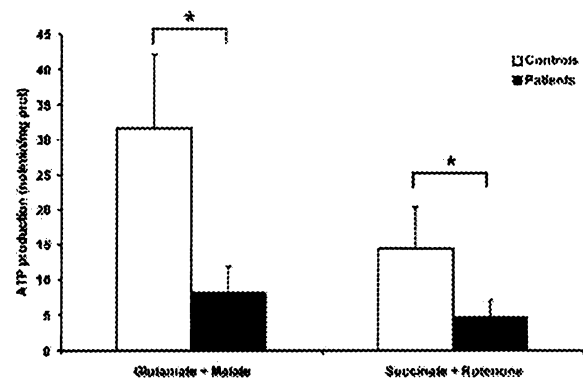

Fig. 1. Increased sensitivity of respiration to aurovertine in ATPase-deficient patient cells. Respiration of intact cells was titrated with aurovertin, specific inhibitor of F_1-ATPase at indicated concentrations. Data are expressed in percentage of basal, non-inhibited cellular respiration and represent mean+SD of analysis of four different patient fibroblasts and controls performed in triplicates. Significance of the difference between patients and control is indicated, *p   0.001.
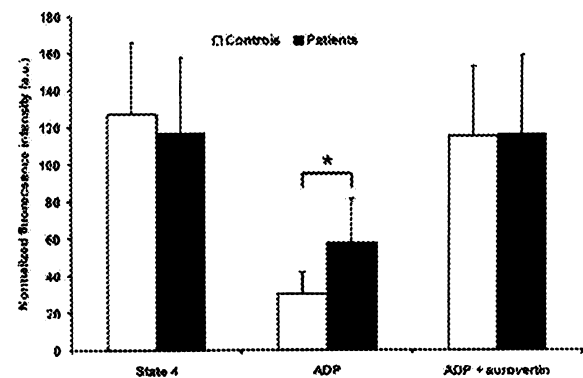

Fig. 3. Changes in mitochondrial membrane potential in ATPase-deficient patient cells. Steady-state values of mitochondrial membrane potential were determined by TMRM cytofluorometry in digitonine-permeabilised cells supplied with 10 mM succinate (state 4), after addition of 1 mM ADP (state 3ADP) and in the presence of ADP and 1 µM aurovertin. TMRM fluorescence was related to MitoTracker Green fluorescence and data represent mean+SD of analysis of four different patient fibroblasts and controls, *p   0.001.

than that by complex I either in forward or reverse electron flow (Miwa et al., 2003; Lambert and Brand, 2004). Also enzymes from the outer mitochondrial membrane (and hence $\Delta\Psi_m$-independent), cytochrome $b5$ reductase and monoaminooxidases have been implicated as ROS producers (Andreyev et al., 2005). The increase in ROS production in our study appears to be smaller than that observed by group of Manfredi in the qualitative ATPase defect using the same probe and analyzing NARP cybrids with T8993G mutation at homoplasmic, 100% mutation load (Mattiazzi et al., 2004). This could imply that the ROS component of the pathogenic mechanism might be more important for degenerative necrotic changes in the brain. On the other hand, both types of studies determine only the apparent level of ROS intermediates that reflects the balance between ROS generation and ROS inactivation by different cellular components of antioxidative defense. Therefore, future parallel studies of these components will be essential before a more precise picture of the entire pathogenic mechanism of different ATPase disorders can be drawn.

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Article 6

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Mitochondrial diseases and genetic defects of ATP synthase

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Abstract

ATP synthase is a key enzyme of mitochondrial energy conversion. In mammals, it produces most of cellular ATP. Alteration of ATP synthase biogenesis may cause two types of isolated defects: qualitative when the enzyme is structurally modified and does not function properly, and quantitative when it is present in insufficient amounts. In both cases the cellular energy provision is impaired, and diminished use of mitochondrial $\Delta\mu H^+$ promotes ROS production by the mitochondrial respiratory chain. The primary genetic defects have so far been localized in mtDNA *ATP6* gene and nuclear *ATP12* gene, however, involvement of other nuclear genes is highly probable.
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1. Introduction

Inherited dysfunction of mitochondrial oxidative phosphorylation system is increasingly recognized as a frequent cause of human disease [1]. Most of the protein subunits of the mitochondrial respiratory chain are encoded by nuclear genes, while only 13, but essential, subunits are encoded by mitochondrial DNA (mtDNA). Mitochondrial energy provision thus uniquely depends on two genomes. Numerous nuclear and mtDNA mutations have been identified in affected patients to cause combined defects as well as isolated disorders of individual oxidative phosphorylation enzymes, including mitochondrial ATP synthase. Most isolated defects of ATP synthase are associated with alterations in the biosynthesis of the enzyme and can be caused by mutations in subunit genes or in ancillary proteins essential for the enzyme assembly. The ATP synthase is a key component of mitochondrial energy conversion in the mammalian organism as it produces most of the cellular ATP in aerobic cells. No wonder that pronounced defects of this enzyme result in mitochondrial diseases which are highly deleterious and manifest primarily in children, very often shortly after birth.

2. ATP synthase biogenesis

Mitochondrial ATP synthase is composed of a catalytic F_1 part connected by two stalks with a membrane-embedded F_0 part. The mammalian enzyme is built of at least 16 different subunits (F_1: $\alpha_3\beta_3\gamma\delta\epsilon + IF1$, F_0: a, b, c_{10}, d, e, f, g, F_6, A6L, OSCP, (factor B)) [2–4] of which two F_0 subunits — the subunit a (subunit 6) and subunit A6L are encoded by mitochondrial DNA (mtDNA). ATP synthase complex is formed stepwise with the assistance of several assembly factors but the mechanism of how the mammalian ATP synthase assembles from individual subunits is still not well understood. In the case of F_1, a close similarity of its structure in all types of energy-transducing membranes suggests an analogous assembly mechanism in mammalian cells, lower eukaryotes and prokaryotes. Concerning the F_0, the situation is complicated by increasing evolutionary complexity of the F_0 structure, which gained 7 new subunits from bacteria to man. Most of the present knowledge of mitochondrial ATP synthase biogenesis originates from studies in yeast but the assembly process in mammalian cell might be modified as there are substantial differences between higher and lower eukaryotes such as the number and location of F_0 subunit c genes, ATP synthase-specific assembly factors, or factors regulating transcription of mtDNA-encoded ATP synthase genes.

It has been shown that F_1 assembles in mitochondria of a yeast cytoplasmic *petite* mutant producing nonfunctional F_0 [5]

as well as in mammalian rho^0 cells , but functional proton channel of the F_0 portion has not been observed in mitochondria lacking the β subunit . Ordered assembly of the yeast F_0 sector was demonstrated using mutants for the mitochondrially encoded subunits . A principal role of subunit 9 (subunit *c*) was suggested as in its absence subunits 6 and 8 (equivalent to A6L) were not associated with the enzyme complex. Sequential addition of mitochondrially-synthesized subunits was proposed to start with subunit *c*, followed by subunit 8 and then subunit 6. Moreover, subunit 6 was absent in cells devoid of any of the stator-stalk subunits: subunit *b* , OSCP and subunit *d* , which might assemble in the order of *b*, OSCP and subunit *d* .

Similarly, in human cells, the incorporation of subunit 6 appears to be at the late stage of enzyme assembly ( ), as shown by the composition of assembly intermediates in cells with doxycycline-arrested mitochondrial protein synthesis , 8993 mutation in *ATP6* gene or diminished content of subunit 6 due to altered processing of the *ATP6-COX3* transcript . Combination of pulse-chase labelling and two-dimensional electrophoretic methods led to the following scheme: assembly starts with the formation of F_1, which then directs a sequential assembly of the membranous part. After the attachment of F_1 to subunit *c*, subcomplexes are formed, involving other nuclear-encoded subunits including subunits *b*, OSCP and F6. The incorporation of mitochondrially encoded subunits 6 and A6L completes the formation of the ATP synthase .

ATP synthase assembly depends on the assistance of multiple proteins that have substrate-specific chaperone-type functions. Altogether 5 factors have been identified in yeast, of which Atp11p and Atp12p mediate the formation of the F_1 moiety via interaction with subunits β and α, respectively (for review see). The role of Fmc1p in F_1 assembly is less clear . Atp10p and Atp22p are essential for the formation of the F_0 part during which Atp10p assists in the incorporation of the subunit 6 . In mammalian cells only the orthologues of yeast *ATP11* and *ATP12* but no F_0-specific assembly factors have been found

( ). Nevertheless, the existence of specific factors involved in mammalian F_0 formation is quite probable.

Further steps in the mammalian ATP synthase biogenesis include generation of dimers with the aid of subunits *e* and *g* , formation of higher oligomers (V1–V4) and possibly also supercomplexes with other inner mitochondrial membrane proteins, e. g. with phosphate and adenine nucleotide carriers in the "phosphorylating assembly" — so called ATP synthasome . Moreover, numerous recent studies identified ectopic location of ATP synthase on the plasma membrane surface of several types of mammalian cells. While the experimental evidence for the functional involvement in intracellular signalling as a receptor for apolipoprotein A-I , angiostatin , or enterostatin and beta-casomorphin1-7 of ectopic ATP synthase in different cell types is quite convincing, nothing is known about the mechanism by which the ATP synthase gets there. It might possibly be transferred from mitochondria as a complete enzyme by membrane fusion.

The cellular content of mammalian ATP synthase relative to other respiratory chain enzymes is rather stable in many tissues, and biogenesis of ATP synthase requires coordination of nuclear and mitochondrial genomes and generally shares the common regulatory cascades with other mitochondrial oxidative phosphorylation complexes . Up- and down-regulation of ATP synthase biogenesis involves both transcriptional and posttranscriptional regulation . The amount of ATP synthase may be controlled by the availability of subunit *c*. This was demonstrated in the thermogenic brown fat where the ATP synthase content was physiologically reduced up to 10-fold due to depressed transcription of subunit *c* gene . It has been further shown that *c* subunit overexpression can restore, at least in part, the ATP synthase content in brown fat. The expression of subunit *c* genes seems to determine the ATP synthase content in other tissues as well . Indeed, the existence of differently organized and regulated isogenes coding for identical subunit *c* would be advantageous for multiple factor regulation of *c* subunit synthesis and thus ATP synthase biogenesis.
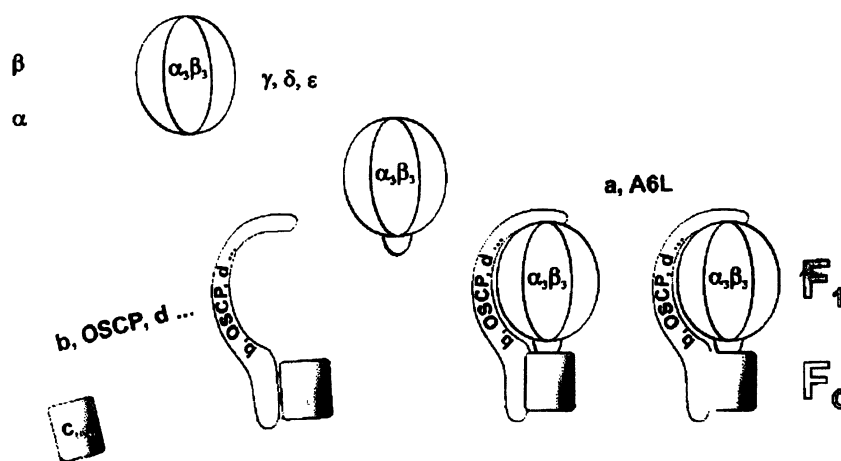

Fig. 1. Assembly of the mammalian ATP synthase. ATP synthase is formed in several successive steps. Assembly of the F_1-part is dependent upon the assistance of specific ancillary factors, Atp11 and Atp12, not belonging to the final complex. In the yeast enzyme, additional factors have been described to be involved in the formation of F_1 (Fmc1p) and F_0 (Atp10p, Atp22p).

3. Qualitative and quantitative defects of ATP synthase

Generalized decrease in the content of human ATP synthase or alteration of its structure and function are associated with severe pathological states resulting in typical mitochondrial diseases. There are two types of isolated deficiency of ATP synthase that have been described, differing in the pathogenic mechanism, biochemical phenotype (structural and functional) as well as in clinical presentation.

The first, and long well-known type, are qualitative defects of ATP synthase caused by mutations in one of mtDNA-encoded ATP synthase subunits — the protonophoric subunit 6 . Maternally transmitted mutations in the *ATP6* gene are heteroplasmic and pathogenic phenotype correlates with their mutation load . Of the five missense mutations described so far, the T8993G mutation that leads to the Leu^{156}→Arg exchange is the most harmful (see). At a high mutation load, up to approximately 95%, it manifests as neuropathy, ataxia, and retinitis pigmentosa (NARP) or as fatal encephalopathy known as Leigh syndrome . Subunit 6 operates together with the ring of subunits *c* as the F_0 proton channel and proton translocation from the intermembrane space to the matrix induces *c* ring rotation that is transmitted to the F_1 moiety, thus driving ATP synthesis . *ATP6* missense mutations prevent ATP synthesis but the reverse reaction, ATP hydrolysis, is possible. On the basis of subunit *a* mutation in bacteria it has been proposed that the H$^+$ translocation is impaired but later on it was shown that NARP cells are capable of ATP-driven H$^+$-translocation and that mutated F_0 can translocate protons from the cytosol to the matrix side of the inner mitochondrial membrane . Therefore, it appears that *ATP6* mutations disturb the intraenzyme coupling of H$^+$-transport with the *c*-ring rotation and ATP synthase is unable to utilize the proton translocation down the membrane proton gradient for the phosphorylation of ADP.

A different form of qualitative ATP synthase deficiency is represented by another type of *ATP6* mutation, microdeletion of TA in position 9205–6 (ΔTA9205). This mutation is very rare,

only two cases having been found to date . In mammalian mtDNA the 5'-part of the *ATP6* gene partly overlaps with the *ATP8* (A6L) gene and the 3'-end is followed by the *COX3* gene. A primary tricistronic transcript is cleaved and polyadenylated to mature *ATP8/ATP6* bicistronic mRNA and *COX3* mRNA. The hypothesis that the mutation removes the STOP codon of *ATP6* and also the cleavage site between *ATP6* and *COX3* transcripts was confirmed in a patient with severe encephalopathy where a several-fold decrease of *ATP6* and *COX3* mRNAs was found. It resulted in diminished synthesis and cellular content of subunit 6, thus producing subunit 6-less enzyme capable of hydrolyzing but not synthesizing ATP. Absence of subunit 6 also interfered with the assembly mechanism leading to the accumulation of incomplete assemblies , similar to those observed in cells with T8993G mutation or cells with doxycycline-inhibited translation of mtDNA-encoded proteins. The primary problem in ΔTA9205 mutation seems to be the tricistronic *ATP8/ATP6-COX3* transcript processing. Interestingly, this was only little affected in the other homoplasmic case , which also differed biochemically and clinically in normal biosynthesis of subunit 6, unaltered mitochondrial ATP production and very mild presentation of transient hyperlactacidemia. This unexplained difference in the manifestation of *ATP6* homoplasmic mutation indicates the existence of a factor, as yet unknown, that is apparently involved in mitochondrial *ATP6-COX3* mRNA processing.

The second currently known type of isolated disorders of ATP synthase are quantitative defects in which the cellular content of the enzyme is selectively reduced to less than 30% of the control, relative to the content of respiratory chain enzymes . The disorder is of nuclear genetic origin, patients do not show any mutations in mtDNA *ATP6* and *ATP8* genes, and ATP synthase content is fully restored by replacement of the nucleus in transmitochondrial cybrids . The biosynthesis of ATP synthase is inhibited but the expression of genes coding for enzyme subunits is not changed, including the level of transcripts of subunit c. Unlike *ATP6* mutations, the ATP synthase-deficient cells do not show accumulation of any incomplete assemblies, and it has been
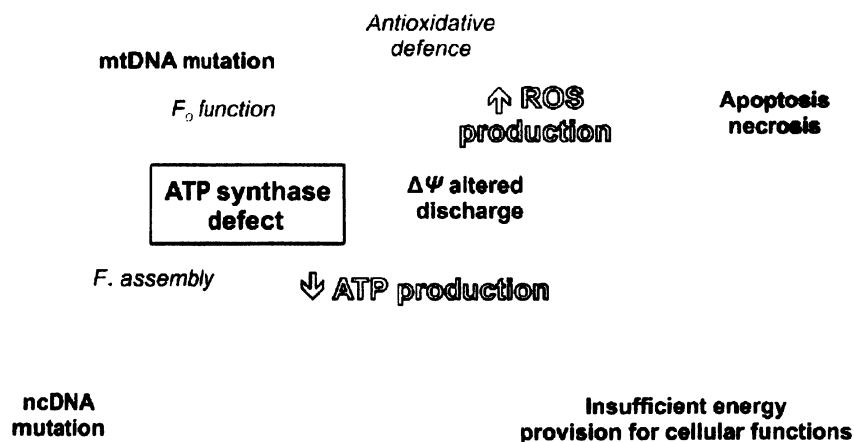

Fig. 2. Pathogenic mechanism of ATP synthase defects — energy deprivation and increased ROS production.

concluded that the problem lies in an early stage of enzyme assembly when the F_1 is formed [49]. At present, more than dozen patients with ATP synthase deficiency have been diagnosed [48–53]. Most of them display a remarkably uniform phenotype with an early onset already in newborns, severe and often fatal hyperlactacidemia, hypertrophic cardiomyopathy and elevated levels of 3-methylglutaconic acid in urine. About half of the patients die within the first days or months of life; however, long survival even for years, is also possible. Degenerative necrotic changes in the brain stem, typical of *ATP6* mutations, are rare. Detailed investigation of F_1 assembly factors showed in one case with pronounced brain atrophy a homozygous TGG-AGG missense mutation in exon 3 of *ATP12* changing Trp^{94} to Arg in the Atp12p assembly factor [53]. However, in others, clinically distinct case mutations in *ATP11* and *ATP12* were excluded, and normal expression of the F_1-assembly genes was found. Interestingly, this contrasts with the situation of ATP synthase-poor brown adipose tissue where *ATP12* mRNA is highly upregulated [54]. Apparently, the quantitative disorders of ATP synthase are caused by different genetic defects that remain to be identified. They may include additional mammalian ATP synthase-specific assembly factors that have not yet been recognized.

4. Two components of the pathogenic mechanism in ATP synthase disorders

Both types of ATP synthase disorders result in a decreased ability of mitochondria to produce ATP in patient cells. This has been clearly shown for *ATP6* mutations as well as for quantitative defects of ATP synthase. In milder mutations of *ATP6*, the ATP synthesis is clearly less affected than in the T8993G patients [14,15,55–58]. Experiments with T8993G cybrids and fibroblasts revealed an up to 5- to 20-fold decrease in ATP production at or near to homoplasmic state. A similarly pronounced decrease has been found in quantitative defects of ATP synthase that display only 10–30% of normal enzyme content. Substrate-supported mitochondrial ATP production in permeabilized cells was decreased to 30% or even more, depending on the substrates [49,51]. Indeed, in both types of ATP synthase disorders the ATP production measurements have been performed in cells that are largely glycolytic, such as cultured fibroblasts, cybrids or blood cells. The key question is how the altered function of ATP synthase affects the ATP production in patient tissues in vivo and how important it is for biochemical and clinical phenotype of the disease. The mainly affected tissues, brain and heart, primarily rely on oxidative metabolism and it is expected that a decline of aerobic energy provision can have deleterious effects on their function and morphology. Studies in mice indicate that due to nonlinear dependence of coupled respiration on ATP synthase content, significant ATP synthesis is to be expected even when the ATP synthase activity is very low. This is apparent from ATP synthase threshold values in different tissues [59,60]. Similar data for human tissues are missing, but even here, there must exist a significant spare capacity of ATP synthase, otherwise the patients with ATP synthase content reduced to 10% of the control would not be able to survive at all.

The low ability of mitochondria to utilize respiration-generated proton gradient for ATP synthesis generally means that the mitochondrial membrane remains hyperpolarized, which may lead to increased generation of reactive oxygen species (ROS) by the respiratory chain [61]. It has been demonstrated that the cells with *ATP6* mutations maintain high values of $\Delta \Psi$ [51,57] and also the cells from patients with quantitative defects of ATP synthase displayed increased $\Delta \Psi$ in state 3 (ADP) as well as in state 4 [49,62]. In both types of ATP synthase disorders, it was also documented that high $\Delta \Psi$ values indeed cause increased ROS production. The T8993G mutation caused an increase in MnSOD and in ROS levels determined with the fluorescent probe DCFDA [57,63]. Similarly, cells with quantitative defects of ATP synthase showed elevated levels of ROS and upregulation of antioxidative defence components [48,62]. Importantly, increased ROS production in both types of disorders was prevented by the uncoupler FCCP, indicating that it was the high membrane potential that upregulated the ROS production.

For a long time, uncompensated ROS production has been implicated in the pathogenesis of human mitochondrial diseases [64]. Now it appears that, besides complex I defects [65–67], the increased oxidative stress represents an important factor of the molecular pathogenic mechanism also in isolated disorders of ATP synthase. Thus, energy deprivation and elevated ROS production are the two major components of the pathogenic mechanism in isolated defects of ATP synthase that are closely related to each other (Fig. 2). At present, it is difficult to say which one is more important for the manifestation of the disease and whether a different proportion between ROS production and decline in mitochondrial ATP synthesis can be responsible for distinct clinical phenotypes in qualitative and quantitative ATP synthase disorders. Nevertheless, different types of antioxidants, such as perfluoro-tris-phenylnitrone, N-acetylcysteine, dihydrolipoic acid or Coenzyme Q_{10}, had an apparent beneficial effect on the level of ROS production, the cell viability, occurrence of apoptotic markers and even ATP production in NARP cells with T8893G mutation [57,63], which makes the ROS production a promising target for the therapy in isolated disorders of ATP synthase.

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Article 7

Comparison of various methods for measurement of mitochondrial reactive oxygen species production in fibroblasts from patients with ATP synthase deficiency using CM-H₂DCFDA.

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Running title:
DCFDA and ROS measurement

Abbreviations:
CM-H₂DCFDA, ROS,

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Abstract

Background

Reactive oxygen species (ROS) have been proposed as one of pathological mechanisms of severe mitochondrial diseases caused by dysfunction of respiratory chain enzymes. However, methods for ROS production measurements are often criticized to give conflicting results. We therefore used the model of ATP-synthase deficiency for comparison of various methodologies for quantification of ROS production with CM-H_2DCFDA.

Methods

We established methods for measurement of in vivo ROS production on confocal microscope (using single- or two-photon excitation) and fluorescence plate-reader. We compared data on ROS production obtained by these methods with respect to absolute and relative values of ROS production and the probe autooxidation and photoactivation.

Results

We found that patient cells produce significantly higher amounts of ROS than controls. The increase was 3-fold when using single-photon microscopy (488 nm laser), 5-fold when using two-photon microscopy (800 nm laser) and 1.5-fold when assessed by plate-reader measurements (485 nm excitation), respectivly. In all cases, uncoupler FCCP similarly diminished the rate to about 30% of its original (coupled) values. For better quantification of differences in mitochondrial ROS production, we also calculated ΔFCCP patient/ΔFCCP control ratio.

Conclusions

All methods tested give principally equivalent results, albeit to lesser or bigger extent influenced by probe photoactivation. When focusing only on mitochondrial portion (FCCP sensitive), the effect of photoactivation is partially eliminated by comparing only differences in production rate.

Introduction

Dysfunction of mitochondrial energy-converting system of respiratory chain enzymes results in severe mitochondrial diseases that affect primarily brain, heart, muscle and other tissues with high energetic demands. The pathogenic mechanism includes two components, energetic deprivation due to insufficient production of mitochondrial ATP and increased ROS production by mitochondrial respiratory chain components (1,2). There are several sites in mammalian respiratory chain proposed as superoxide producers: Complex I, Complex III and mGPDH. Even under normal physiological conditions, the leak of electrons results in production of superoxide anion and subsequent formation of other radical intermediates (3). Up to now, it has been clearly shown that ROS production can be increased in mitochondrial diseases associated with Complex I defects (4) and with dysfunction of mitochondrial ATP synthase (1,5). In the first case, the structural and functional alterations of the subunits within the complex I lead to increased electron leak and formation of ROS (4). In the second case, the increased ROS production appears to be caused by the elevated values of mitochondrial membrane potential $\Delta\Psi_m$, the key modulator of mitochondrial ROS formation (6). The primary cause of the higher $\Delta\Psi_m$ can be either the structural change of the ATPase due to mutations in one of the subunits encoded by mtDNA (5,7,8), or insufficient biosynthesis of ATP synthase associated with the defects in specific assembly factors that are encoded by nuclear DNA (1). In both cases, the impaired function of ATP synthase results in altered discharge of mitochondrial proton gradient (1).

The exact role of oxidative stress in mitochondrial pathology is still poorly understood. For a long time, the isolated mitochondria have been used to study ROS production. However, this simplified system can provide us only limited information about the situation under in vivo conditions. Studies on intact living cells are therefore of primary interest. For demonstration of increased oxidative stress in different ATPase defects 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) was used as probe to asses the extent of ROS production in intact cells (1,5). Although the phenomenon was clearly observable both in cells with mutated mtDNA (subunit ATP6) and in cells with 70-90% decrease of ATPase content, the relative extent of ROS production was quite different. We therefore decided to compare various methodologies used for quantification of ROS production with CM-H$_2$DCFDA.

Dichlorodihydrofluorescein (H_2DCFDA) and its derivatives (Carboxy-H_2DCFDA, CM-H_2DCFDA) are widely used as probes for measurement of redox activity in intact cells (9). DCFDA is oxidized by hydroxyl, peroxyl and alkoxyl radical and peroxynitrite, while neither H_2O_2 nor $O_2^{\cdot-}$ can directly oxidize it (10-12). Transition metal ions or cellular heme proteins such as peroxidase must catalyze reaction with H_2O_2. Due to this broad spectrum of detected ROS and non-trivial reaction mechanism, the data should be interpreted more as a measure of cellular redox state than as a intensity of any particular ROS production. Probably due to this low specificity , there is quite big debate about reliability of data obtained by this method (13). Many parameters have been proposed to influence the rate of H_2DCFDA cleavage and oxidation, such as intracellular iron concentration (14), cellular peroxidase level or heme protein content (11,12). Most prominent discussions were raised about the effect of photoactivation and potential phototoxicity of this probe (15,16).

In this work, we used fibroblasts derived from patients with isolated ATP synthase defect to study ROS production in vivo and its dependence on energetic state of the mitochondrial membrane.

We established methods for measurement of ROS production with CM-H_2DCFDA, using either single- or two-photon excitation on confocal microscope with the possibility to relate both signals to mitochondrial mass, determined by mitochondria selective probe MitoTracker DeepRed. For high-throughput determination of ROS production in intact cells, we also used fluorescence plate-reader. We tried to compare data obtained by these three methods with respect to absolute and relative values of ROS production and the intensity of probe autooxidation and photoactivation.

Materials and Methods

Patient

Skin fibroblasts from patient with isolated deficiency of mitochondrial ATPase due to selectively decrease biosynthesis of the enzyme were used (17). The defect is of nuclear origin but the mutated gene is still unknown. Biochemically, the ATPase content and

activity are decreased to <30% while other respiratory chain enzymes are unaffected. Clinical symptoms are early onset, fatal lactic acidosis, cardiomyopathy and hepatomegaly.

Cell cultures

Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium. For confocal microscopy measurements, cells were grown on round 2.5 cm in diameter coverslips. Each coverslip was mounted between two steel rings forming a chamber designed for live cell imaging. In the case of fluorometric detection of hydrogen peroxide production, cells were grown in multi-well culture plates to confluence.

Confocal microscopy

Intact fibroblasts from controls or patients were preloaded in dark for 20 min in HEPES buffer (135 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.4) with 1 μM CM-H$_2$DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, Molecular Probes) and 50 nM MitoTracker DeepRed (Molecular Probes). Increase in green fluorescence serves as a measure of oxidative activity in cell, as non-fluorescent CM-H$_2$DCFDA is cleaved by cellular esterases after upload into the cell to non-fluorescent CM-H$_2$DCF and than oxidized by ROS to fluorescent dichlorofluorescein (DCF). Measurements were done on Leica TCS SP2 and Zeiss LSM 510 confocal microscopes. Probes were excited by 488 nm or 633 nm lasers respectively. Settings were as follows: Leica - laser powers 4% or 9% for 488 or 633 nm laser respectively to ensure low level of photoactivation. Emission wavelengths 500-533 nm for green fluorescence of CM-DCF and 673-725 nm for red fluorescence of mitochondria localized MitoTracker DeepRed using Leica's AOBS system. Zeiss - laser powers 0.5% or 7% for 488 or 633 nm laser respectively, emission filters were band-pass 505-550 nm for green and long-pass 650 nm for red channel.

Two-photon confocal microscopy

Cells were prepared in the same way as for one-photon microscopy. Two-photon laser Mira + Verdi (Coherent) tuned at 800 nm wavelength with the intensity of 2 mV measured at the level of objective was used for probe excitation. Emission was collected between 500 and 533 nm for green fluorescence of DCF, as in the case of single photon excitation.

Fluorescence plate-reader

For measurement, culture medium on multiwell-plates was changed for HEPES buffer with 1 μM CM-H₂DCFDA. The formation of the fluorescent compound, DCF, was monitored at an excitation wavelength of 485 nm (bandwidth 15 nm) and an emission wavelength of 535 nm (bandwidth 30 nm) using a Wallac Victor^2 Multilabel Counter.

Data analysis

Confocal time series images were analyzed with the help of Leica Confocal Software (Leica Microsystems, Germany). The intensity of fluorescence was measured in multiple regions of interest (ROI), representing individual cells and data were exported into MS Excel (Microsoft, USA). Data from single photon images were subsequently smoothed by adjacent averaging with window width of 20 frames. Presented data are averages of normalized values with expressed standard deviation.

Fluorometric data were analyzed in MS Excel (Microsoft, USA). Measured value for each well represents mean of four measurements made in various points of the well with displacement between points to be set at 4 mm.

Results

Microscopy

First, we compared usage of one-photon vs. two-photon lasers for DCF excitation in confocal microscopy. We set-up the same initial conditions for both measurements: intact fibroblasts grown on coverslips were preloaded for 20 min with 1 μM CM-H₂DCFDA and 50 nM MitoTracker DeepRed in HEPES buffer in tissue culture thermostat (37°C, 5% CO₂ in air). Then the coverslips were mounted into AttoFluor chambers and 1 ml of HEPES buffer with fluorophores was added. Chambers were transferred to the thermostated microscope stage (37°C) and allowed to equilibrate for another 10 min. Subsequently we located field with enough cells and started measurements. Figure 1 summarizes data obtained with 488 nm laser, while Figure 2 shows data from two-photon laser.

As could be seen, the advantage of using 488 nm excitation wavelength on our system is the possibility to simultaneously monitor mitochondrial mass with MitoTracker DeepRed dye excited by 633 nm laser. This probe is the best from mitochondria targeting probes, as it displays only very low spectral overlap with DCF fluorescence. Usage of MitoTracker offers the possibility to correlate ROS signal to mitochondrial mass (compare

graphs with correlated and uncorrelated data Figure 1 B and insert). This is not possible in the case of two-photon laser, as our system does not allow simultaneous imaging with single and two-photon lasers and MitoTracker DeepRed cannot be efficiently excited by two-photon laser.

On the other hand, in the case of 488 nm excitation photoactivation of CM-H$_2$DCF is higher, what is evident from exponential character of the curve especially in patient cells. Photoactivation effect makes impossible to evaluate the effect of uncoupler in single experiment. Instead, cells had to be either pretreated with FCCP during CM-H$_2$DCFDA loading or left without FCCP and imaged in two independent experiments. When using two-photon laser the increase in DCF fluorescence was linear for at least 30 min and the effect of FCCP addition could be followed during one experiment (Figure 2B).

Fluorescence-plate reader

To develop method for high-throughput quantitative screening of ROS production in different patients' cells available, we switched from microscope to fluorescent plate-reader. Here we used cells cultivated to confluence on 24 well plates. Cells were incubated for 2 hours in medium with CM-H$_2$DCFDA and fluorescence was measured at t = 0 and t = 2 h. Fluorescence at t = 0 was subtracted as background. Data from control and patient cells are summarized in Figure 3. Direct comparison of the data from patient fibroblasts obtained by confocal microscopy and plate-reader shows differences in the rate of ROS production. This could be explained by the still present effect of photoactivation in the case of confocal microscopy. In plate-reader measurements, the cells are exposed to excitation light just twice, while microscope measurement represents time-series of 150 image acquisitions.

ROS production of mitochondrial origin

Relative rates of ROS production as determined by individual methods are summarized in the Table 1., The rate of ROS production measured with 488 nm laser was about 3-times higher in patient cells than in control cells. For comparison, data from two confocal systems (CSLM1 and CSLM2) are included in the table. As could be seen, these data match quite well to each other, even that different settings were used on those systems. When assessed by two-photon microscopy, ROS production rate in patient cells was 5-times higher than in control fibroblasts. Lowest, but still significant, difference (1.5 the control rate in patient cells) was found by plate-reader measurements. After FCCP addition, the rate in patient cells was diminished similarly in all types of measurements.

indicating that about one third of the signal was insensitive to FCCP and thus not of mitochondrial origin (26 – 43 % using different methods).

The apparent rates of ROS production observed might include both the intra- and extramitochondrial components. Because mitochondrial ROS production is highly sensitive to uncouplers (6,18), we performed also measurements in the presence of FCCP to quantify the mitochondrial portion of ROS production. We investigated the effect of uncoupling $\Delta\Psi_m$ with increasing amounts of FCCP. As could be seen from Figure 4, intensity of ROS production is rapidly decreased even by low (5 nM) concentration of FCCP, both in control and patients cells. Apparently, the high mitochondrial membrane potential ($\Delta\Psi_m$) in ATPase-deficient cells, caused by defective discharge of proton gradient by ATP synthase seems to be responsible for accented ROS production.

Interestingly, in all types of measurement the residual value was somewhat higher than the basal rate in controls, which could be related to either the extramitochondrial ROS production or the mitochondrial, which is not associated with the inner mitochondrial membrane.

For better quantification of differences in mitochondrial ROS production among individual cell lines, the use of Δ FCCP patient / Δ FCCP control ratio thus appears to be very promising (Table 1). This should represent real difference between mitochondrial (and hence FCCP sensitive) ROS production in those cell lines. We calculated this ratio for the case of CLSM and plate-reader, i.e. methods, where measurement conditions with FCCP preincubation were used. We found this ratio to be very similar in both cases, seemingly eliminating the effect of probe photoactivation. Both approaches thus indicate that there is 2-2.2-fold increase in mitochondrial ROS production in patient cells.

Discussion

Many researchers consider reactive oxygen species as an important player in pathology of mitochondrial diseases (19). Although many indirect data exists (20-22), there can be also found arguments speaking against it (23). It is therefore of essential importance to obtain reliable and quantitative data about ROS production in intact cells either from tissue culture or bioptic material. In this study, we examined fibroblasts from patients with isolated deficiency of ATP synthase and compared various methods of measurement.

By the measurement of in vivo ROS production, we clearly demonstrated enhanced levels of oxidative stress in fibroblasts of patient with deficiency of ATP synthase.

Intensity of ROS production is sensitive even to mild uncoupling with FCCP. High steady-state levels of $\Delta\Psi_m$ might therefore be responsible for increased ROS production in patients' mitochondria (6). However, for better understanding of the contribution of oxidative stress to entire pathological mechanisms, it is essential to know the real amount of ROS produced in cells.

Photoactivation represents serious problem in all fluorescent measurements and especially in the case of DCF it must be taken into consideration. Our data clearly indicate that usage of 488 nm laser for probe excitation on confocal microscope results in quite pronounced photoactivation, even under low laser powers. Considerably better results gave two-photon laser excitation at 800nm. Data obtained with two-photon excitation show almost linear increase of the signal, when no uncoupler is added. This might be considered as an indicator that photoactivation does not represent serious problem in the latter type of measurements.

All three methods (CLSM, two-photon LSM or fluorescence plate-reader) detected the difference between control and patient cells. Quantification of the data, however, revealed differences between these three methods. Main cause is probably the photoactivation of DCF probe. All methods are plausible for ROS measurements on intact cells with superior sensitivity being the main advantage of microscopy and high-throughput screening of plate-reader.

Major portion of ROS produced by mitochondrial respiratory chain has been shown to be dependent on both components of proton-motive force (PMF) - $\Delta\Psi_m$ (18) and ΔpH (24). Dissipating of PMF by uncoupler, such as FCCP, may therefore help to reveal this mitochondrial component of ROS production. Although, strictly speaking, this does not represent the whole of mitochondrial ROS production, as also enzymes from outer mitochondrial membrane (and hence PMF independent) cytochrome $b5$ reductase and monoamin oxidases have been implicated as ROS producers (25), it represents the traditionally viewed mitochondrial ROS, i.e. those derived from respiratory chain. As we have shown here, evaluating this component may help in eliminating of unspecific DCFDA oxidation and by calculating ratios of FCCP sensitive portions we can even to some extent eliminate the effect of photoactivation.

Another important aspect of ROS production measurements is that all values obtained represent the portion, which has not been readily eliminated by ROS detoxifying mechanisms. Fluorescent probe competes for radicals with vast amounts of scavenging molecules, therefore bringing information more about equilibrium between ROS

production and elimination in a cell than about net ROS production. All such measurements should therefore be accompanied by studies of these scavenging pathways (25).

Acknowledgements

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Tables

	CLSM 1	CLSM 2	2-photon LSM	
	Plate-reader			
Control	1.0	1.0	1.0	1.0
Control + FCCP	0.3	0.15	-	0.47
Patient	2.7	3.06	5.5	1.5
Patient + FCCP	1.18	1.17	2.0	0.4
Δ FCCP Patient / Δ FCCP Control	2.1	2.2	-	2

Table 1: Relative rates of ROS production assessed by confocal microscopy, two-photon LSM or fluorescence plate-reader. Values are relative to controls.

Legend to figures

Fig 1: ROS production analysed by confocal microscopy.
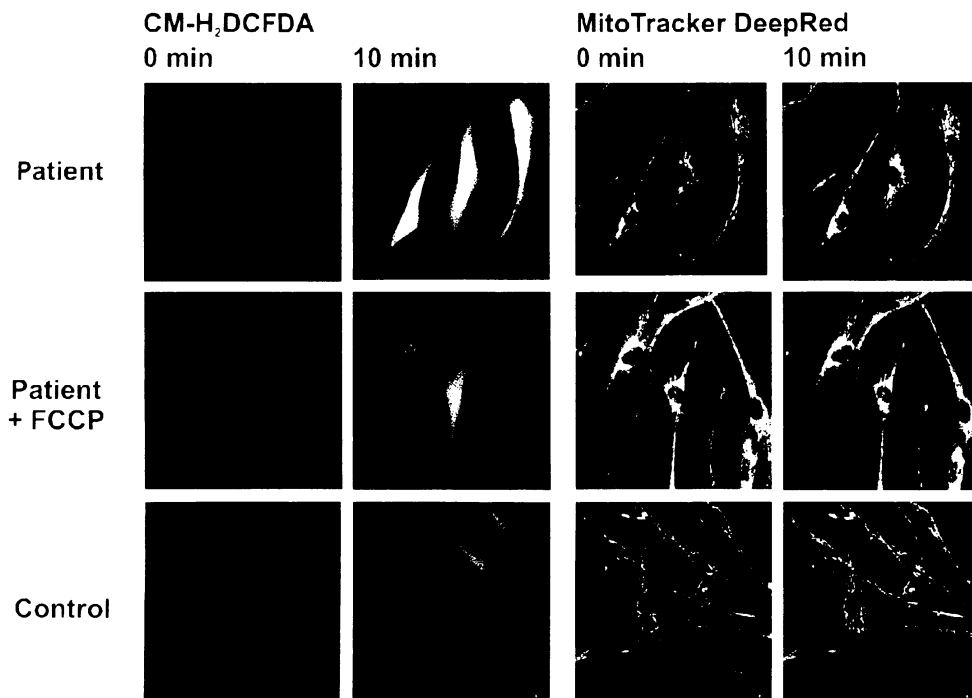
Fig. 2: Increased ROS production by ATP⁻ fibroblasts - analyses by two-photon confocal microscopy using CM-H$_2$DCFDA probe.

Fig. 3: ROS production analysed by fluorescence plate-reader. Uncoupler of oxidative phosphorylation (FCCP, 1 µM) dissipates mitochondrial membrane potential and leads to decrease of ROS production.

Fig. 4: Production of ROS in intact cells is dependent on $\Delta\Psi_m$. FCCP titration of ROS production for patient and control cells.
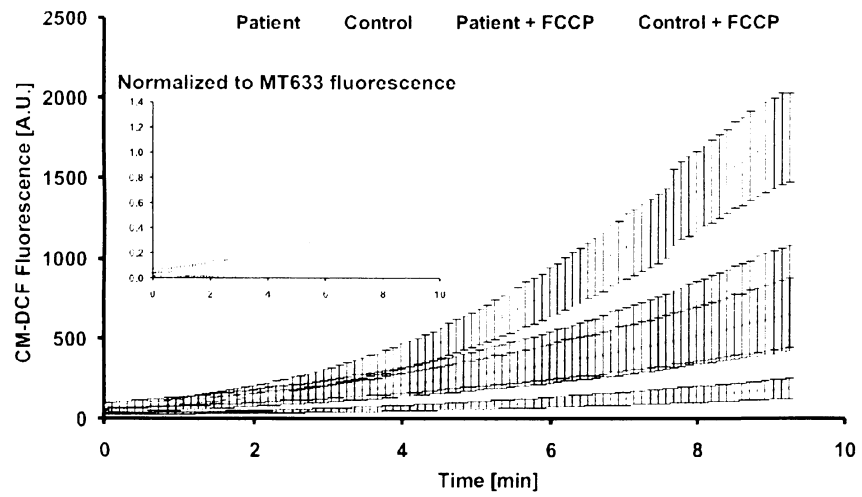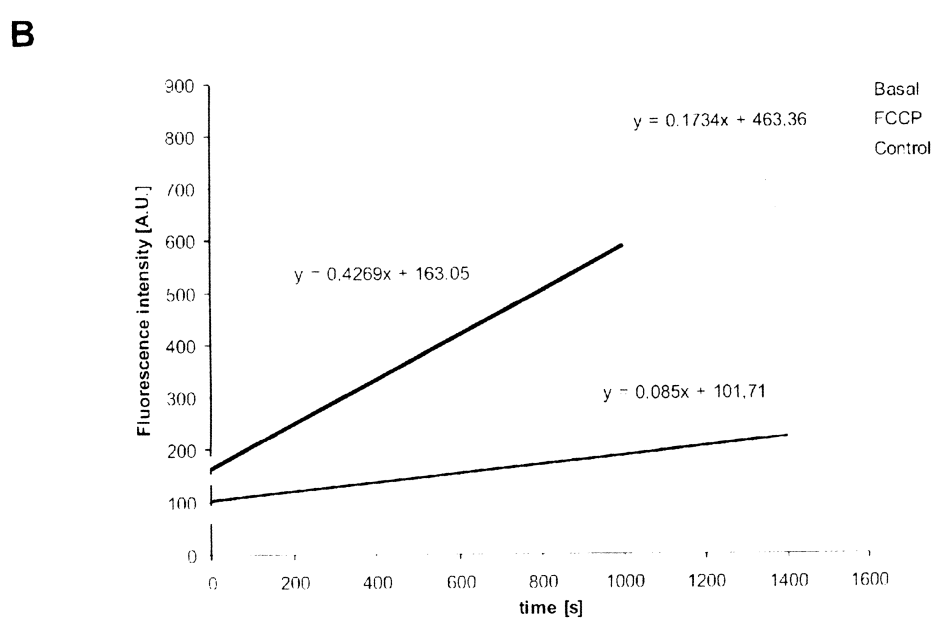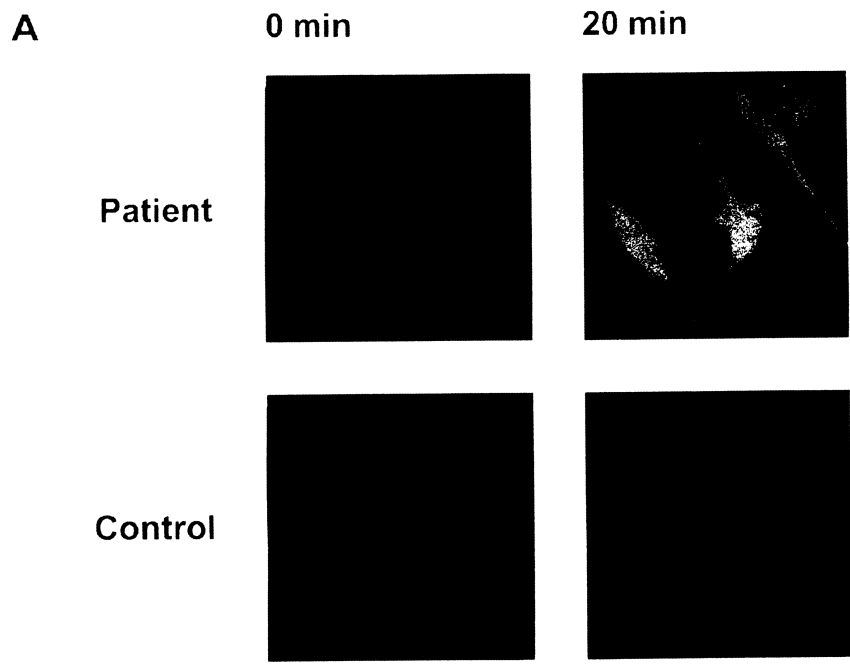
Fig. 1

A

	CM-H$_2$DCFDA		MitoTracker DeepRed	
	0 min	10 min	0 min	10 min
Patient				
Patient + FCCP				
Control				

B

Patient   Control   Patient + FCCP   Control + FCCP

Normalized to MT633 fluorescence

14

Fig. 2

A

	0 min	20 min
Patient		
Control		

B

$y = 0.1734x + 463.36$ — Basal, FCCP, Control

$y = 0.4269x + 163.05$

$y = 0.085x + 101.71$

Fluorescence intensity [A.U.] vs. time [s]

15

Fig. 3

Fig. 4