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Regulation and Disorders of Mammalian Cytochrome *c* Oxidase

Regulace a poruchy savčí cytochrom *c* oxidázy

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Contents

ABSTRACT

Cytochrome *c* oxidase (COX) represents the terminal enzyme complex of respiratory chain metabolic pathway and it occurs as monomer, dimer or as a part of respiratory supercomplexes in the inner mitochondrial membrane. COX assembly process is complicated, highly regulated and depends on many ancillary proteins. Mutations in COX subunits, which are encoded by mitochondrial and nuclear DNA, or in genes encoding its assembly proteins are frequent cause of very severe mitochondrial disorders. SURF1 assembly protein participates in the first steps of COX assembly, but its exact function is not yet clarified. In humans, mutations of *SURF1* gene lead to severe COX defect and fatal neurodegenerative disorder, Leigh syndrome. Knockout of *SURF1* gene in mouse causes isolated COX defect as well, but less pronounced and without involvement of CNS. The aim of the thesis was detailed analysis of disturbed COX biogenesis in a condition of *SURF1* gene mutations or *SURF1* gene knockout, from assembly of COX monomer to interaction of COX into supercomplexes, and to the impact of isolated COX defect on other OXPHOS complexes. Mutations of *SURF1* gene in patient's fibroblasts led to marked accumulation of COX assembly intermediates and to a defect in formation of functional COX monomer, which was preferentially built into an I-III_2-IV_1 supercomplex. Consequently, COX deficiency led to increased amount of OXPHOS complexes I, III and V. In *SURF1*$^{-/-}$ mouse, COX defect was markedly tissue specific. The most pronounced decrease of COX was in mouse fibroblasts, but less marked than in *SURF1* patients fibroblasts. In *SURF1*$^{-/-}$ mouse, the COX monomer was also more stable, interacted much less into supercomplexes and COX assembly intermediates were faster depleted than in *SURF1* patients.

The study of another defect of COX biogenesis due to the unique 9205delTA mtDNA microdeletion of *ATP6*/*COX3* genes was focused on different manifestation of the defect in patients and showed, that the reason is heteroplasmy of mtDNA mutation and steep threshold effect. The pathological phenotype thus manifests when more than 90% of mtDNA becomes mutated.

The last part of the thesis was focused on possible interactions of COX in respiratory supercomplexes with FAD-dependent dehydrogenases. We have found that succinate dehydrogenase as well as glycerol-3-phosphate dehydrogenase form higher molecular weight complexes, which were rather oligomeric and without any involvement of COX.

Key words: Cytochrome *c* oxidase, Leigh syndrome, *SURF1* gene, SURF1 protein, *SURF1*$^{-/-}$ knockout mouse, respiratory supercomplexes, 9205delTA microdeletion

ABSTRAKT

Cytochrom *c* oxidáza (COX) je koncovým enzymovým komplexem dýchacího řetězce a vyskytuje se ve vnitřní mitochondriální membráně jako monomer, dimer a ve formě respiračních superkomplexů. Asemblační proces COX je komplikovaný, vysoce regulovaný a závisí na mnoha pomocných proteinech. Mutace COX podjednotek, kódovaných mitochondriální nebo jadernou DNA, nebo mutace v genech pro COX asemblační proteiny jsou častopu příčinou závažných mitochondriálních onemocnění. SURF1 protein je zapojen do počátečních fází tvorby COX, ale jeho přesná funkce není objasněna. Mutace lidského *SURF1* genu vedou k těžkému defektu COX a fatálnímu neurodegenerativnímu onemocnění, Leigh syndromu. Knockout *SURF1* genu u myši způsobuje také izolovaný COX defekt, ale méně výrazný a bez postižení CNS. Cílem práce byla detailní analýza narušené COX biogeneze vyvolané mutací nebo knockoutem *SURF1* genu, od tvorby COX monomeru a vestavění COX do superkomplexů až po ovlivnění ostatních OXPHOS komplexů izolovaným defektem COX. Mutace *SURF1* genu ve fibroblastech pacientů vedly k výrazné akumulaci asemblačního intermediátu COX a defektu tvorby funkčního COX monomeru, který se preferenčně vázal do I-III_2-IV_1 superkomplexu. COX deficience následně vedla ke zvýšení obsahu OXPHOS komplexů I, III a V. U *SURF1*$^{-/-}$ myší byl COX defekt výrazně tkáňově specifický. Největší pokles COX byl ve fibroblastech, ale mnohem menší než ve fibroblastech *SURF1* pacientů. COX monomer byl u *SURF1*$^{-/-}$ myší také stabilnější, integroval se mnohem méně do superkomplexů a COX asemblační intermediáty se rychleji odbourávaly ve srovnání s fibroblasty *SURF1* pacientů.

Studium dalšího defektu biogeneze COX na podkladě unikátní 9205delTA mtDNA mikrodelece *ATP6*/*COX3* genu bylo zaměřeno na výrazně rozdílnou manifestaci defektu u pacientů. Ukázalo, že příčinou je heteroplasmie mtDNA mutace a prahový efekt se strmým nástupem patologických změn při více než 90% podílu mutované mtDNA.

Poslední část práce byla zaměřena na možné interakce COX v respiračních superkomplexech s FAD-dependentními dehydrogenázami. Jak v případě sukcinát dehydrogenázy, tak glycerol-3-fosfát dehydrogenázy, jsme detekovali vysokomolekulární nativní formy dehydrogenáz, které však byly spíše oligomerní a bez přítomnosti COX.

Klíčová slova: Cytochrom *c* oxidáza, Leigh syndrom, *SURF1* gen, SURF1 protein, *SURF1*$^{-/-}$ myší knockout, respirační superkomplexy, 9205delTA mikrodelece

1. INTRODUCTION

1.1 Cytochrome *c* oxidase (COX)

Cytochrome *c* oxidase (COX) represents the terminal enzyme (complex IV) of the respiratory chain (RC), which catalyzes transfer of electrons from reduced cytochrome *c* (cyt *c*) to oxygen molecule bound in COX structure. The oxygen reduction process is coupled with translocation of four protons through the COX across the inner mitochondrial membrane (IMM) against an electrochemical gradient via three different proton conduction pathways, the D, K and H channels [1,2]. The free energy of the oxidase reaction is thus stored in a proton gradient.

Mammalian COX is composed of 14 protein subunits and in the IMM it occurs in a form of monomer, dimer or in respiratory supercomplexes, by interacting with RC complexes CI and CIII, respectively. Three mtDNA encoded subunits (COX1, COX2, COX3) form the structure of the catalytic core of the enzyme, which contains the binding site for cyt *c* and the redox centers Cu_A (COX2), heme *a* and the oxygen-binding binuclear center heme a_3-Cu_B (COX1) (Fig. 1) [3]. Remaining subunits encoded by nuclear DNA (COX4, COX5a/5b, COX6a/6b/6c, COX7a/7b/7c, COX8) were added to COX structure during eukaryotic evolution. Seven of them contain a single transmembrane helix each (COX4, COX6a, COX6c, COX7a/7b/7c, COX8), extramembrane part of the enzyme is then formed by COX6b at the cytosolic and COX5a/5b at the matrix side [4]. Recently, the NDUFA4 protein, formerly described as CI subunit, was recognized as the 14^{th} nuclear encoded subunit of the COX [5]. These nuclear DNA encoded subunits do not participate in electron transfer, but they are responsible for structural integrity of the enzyme, its regulation and also dimerization, where COX6a and COX6b subunits stabilize dimeric COX by forming the major contacts between the two monomers [6]. As a part of crystalline bovine heart COX structure, 11 molecules of phospholipids were found [7], to which belongs also 3-4 molecules of tightly bound cardiolipins [8].

COX is the only complex of mammalian OXPHOS with known tissue-specific isoforms of nuclear encoded subunits that emphasize the key role of COX in OXPHOS adaptation and regulation in different tissues [4,9]. So far, isoforms for at least 5 subunits (COX4, COX6a, COX6b, COX7a and COX8) have been described in mammals.

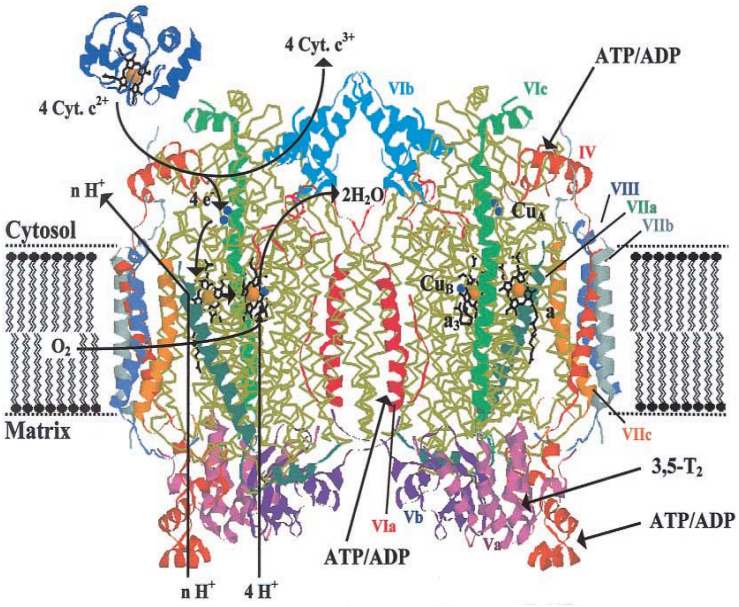

Figure 1. Structure of the dimeric cytochrome *c* oxidase complex from bovine heart (adapted from [4]). *The crystal structure of a cytochrome c molecule (Cyt. c) is shown in dark blue ribbons on the cytosolic side. Marked in yellow backbone are subunits COX1, COX2 and COX3, the nuclear encoded subunits are shown as ribbons in the indicated color and denoted on the right COX monomer in Roman numerals. The hemes c, a, and a_3 are indicated in black; the iron atoms as orange points; Cu_A and Cu_B as blue points. On the cytosolic side on the left COX monomer, the chemical reaction is indicated schematically. On the right COX monomer, the binding sites for the regulatory compounds ATP or ADP and 3,5-diiodothyronine (3,5-T2) are indicated.*

1.1.1 COX assembly process

Biogenesis of mammalian COX is a complicated and highly regulated process that proceeds in sequence through four/five distinct assembly intermediates S1 - S4 (Fig. 2) [10]. The COX assembly mechanism is not spontaneous, because numerous accessory nuclear DNA encoded proteins are needed to build the holoenzyme. Their function is required for all steps of the process and many of them are significantly conserved from yeast to humans [11]. Described and investigated COX assembly proteins in humans are essential for (*i*) regulation of expression of catalytic core subunits (LRPPRC, TACO1, hCOA3, COX14, MITRAC7) [12-16], (*ii*) copper metabolism and insertion (COX17, SCO1, SCO2, COX11, COX19, COA6, COX20) [17-23], (*iii*) heme *a* biosynthesis and insertion (COX10, COX15, FDX2) [24-26], and (*iv*) membrane insertion and processing of catalytic core subunits (OXA1l, COX18) [27,28]. A few other COX assembly proteins have been identified participating in early stages (SURF1, COA5) [29,30] or intermediate stage (PET100, MCUR1) [31,32] of COX biogenesis, but their precise function is not known yet.

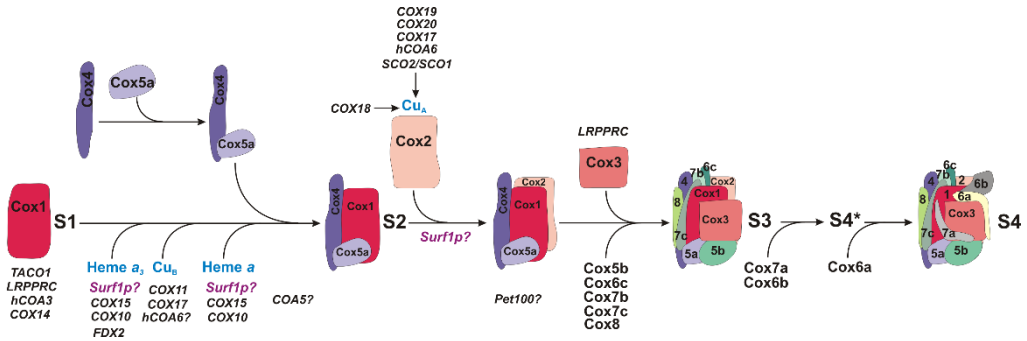

Figure 2. Assembly scheme of human COX
Step-by-step forming of COX through distinct assembly intermediates (S1-S4) from 13 individual COX subunits. S4 corresponds to a fully assembled COX monomer. Accessory assembly proteins are inscribed with italic, question marks mean unknown precise function.

SURF1 assembly protein

SURF1 protein (SURF1) is a 30 kDa hydrophobic protein embedded in the IMM with two predicted transmembrane domains and its central loop facing the mitochondrial intermembrane space [33,34]. The protein has a characteristic N-terminal mitochondrial targeting sequence. The *SURF1* gene is ubiquitously expressed, whereas tissue specific expression was investigated in humans, when e.g. expression in brain was found low compared with other highly aerobic tissues, such as heart, skeletal muscle and kidney [33]. SURF1 is not absolutely required for the COX assembly and appears to increase the efficacy of COX assembly [35-37]. SURF1 is supposed to be involved in a formation of S2 assembly intermediate and in a phase of association of COX2 subunit with COX1-COX4-COX5a subassembly [38,39], but its function might be more redundant, because studies on yeast homologue Shy1 indicate, that Shy1/SURF1 might play a role in heme *a* transfer/insertion into COX1 subunit [40,41].

1.2 COX in respiratory supercomplexes

Individual RC complexes can physically interact and form dynamic supramolecular organizations called supercomplexes (SCs). They were proposed to have many beneficial effects on OXPHOS functioning, including reduced formation of reactive oxygen species, assembly/stabilization of RC complexes and enhancement of the cell respiration through substrate channeling [42-45]. Specific associations between RC complexes can be usually revealed by blue native polyacrylamide gel electrophoresis (BN PAGE), or other types of colorless-native PAGEs [46] when analyzing mitochondrial proteins solubilized by mild non-ionic detergent digitonin [42,47]. This technique enabled characterization of many types of SCs like I-III_2 SC including complex I (CI) and dimeric complex III (CIII), III_2-$IV_{1–2}$ SCs consisting of dimeric CIII and one or two copies of complex IV (CIV), large I-III_2-$IV_{1–4}$ SCs (respirasome) comprising CI, dimeric CIII and one to four copies of CIV and also dimeric ATP synthase, which constitutes oligomeric chains in IMM cristae [48].

SCs are reproducibly isolated together with individual RC complexes. Different ratios among individual complexes and SCs are cell-type specific and respond to physiological stimuli. Therefore, the „plasticity model“ of the OXPHOS system has been proposed to

demonstrate its structural variability - coexistence of fluid structures and SCs. This integrated model reflects the stoichiometry of the complexes and the variable stability of different free/associated structures under different physiological conditions and substrate availabilities in the cell [49,50].

1.3 COX disorders

Mitochondrial diseases caused by COX defects belong to the most severe ones, which can affect single or multiple organs [51,52]. Mutations in the mtDNA genes encoding COX subunit are relatively rare and present with variable clinical phenotypes ranging from isolated myopathy to multisystem disease and with onset from late childhood to adulthood. In contrast, the most of isolated COX deficiencies are caused by mutations in nuclear-encoded genes and are inherited as autosomal recessive disorders. Generally they have an early onset and a fatal outcome. The first reported COX deficiency was published in 1977 and represented the patient with the typical subacute necrotizing encephalomyelopathy called Leigh syndrome (LS) [53]. Over the time, the LS alone or associated with cardiopathy, hepatophaty or nephropathy was found as the most common neurological disorder linked with COX defects.

1.3.1 *SURF1* gene mutations in humans

Mutations in the *SURF1* gene were found to be the most frequent cause of COX deficiency manifesting as typical autosomal recessive LS. The *SURF1* gene is located on chromosome 9q34 in a highly conserved surfeit cluster of six housekeeping genes (*SURF1-SURF6*) and is ubiquitously expressed [54,55]. It consists of nine exons spanning almost 5000bp and codes for 300 amino acid SURF1 protein. The LS manifests usually several months after birth as a fatal neurodegenerative mitochondrial disorder, which was firstly described by Leigh [56] many years before LS was associated with various dysfunctions of mitochondrial RC and energetic metabolism.

Up to now, 74 known *SURF1* gene mutations linked to LS and atypical LS have been summarized [57], but without genotype-phenotype correlation [58-62]. Approximately 22% (16/74) of *SURF1* gene mutations are located in exon 8 suggesting an important function for this region. Mutations in the *SURF1* gene have been subdivided into three groups: insertion/deletion mutations, missense/nonsense mutations and mutations in splicing sites [63]. The most common mutation among reported cases is the deletion c.845_846delCT in

Slavonic population and the deletion c.312_321del10insAT in non-Slavonic population [64,65].

1.3.2 *SURF1*$^{-/-}$ mouse model

To better understand the pathophysiological mechanisms of *SURF1* gene mutations and the function of SURF1 protein in COX biogenesis, the group of Zeviani has generated knock-out mice (*SURF1*$^{-/-}$) model by the insertion of *loxP* sequence in exon 7 to disrupt last portion of *SURF1* gene [35]. These *SURF1*$^{-/-}$ mice failed to show spontaneous neurodegeneration at any age and were also protected from neuronal damage induced by kainic acid, which was accompanied by reduced mitochondrial uptake of calcium ions. These animals were smaller at birth, had mild reduction in motor skills at adult age and COX activity was found to be mildly reduced in brain, skeletal muscle, heart and liver tissues. Interestingly, *SURF1*$^{-/-}$ mice showed prolonged lifespan, about 5 months longer compared to wild-type littermates.

2. AIMS OF THE THESIS

Cytochrome *c* oxidase (COX) is one of the key enzymes of mitochondrial energetic metabolism and its function is regulated by set of metabolites in response to different physiological conditions. Defects of COX in humans lead to a wide variety of disorders with severe pathological features and can arise from mutations in its mtDNA or nuclear DNA encoded subunits or from mutations in any of the assembly factors. For several isolated COX deficiencies, mouse models were created to better understand the pathophysiological mechanisms of these diseases.

The aim of this thesis was to characterize in detail molecular pathogenic mechanism of COX deficiency at the level of COX biogenesis and its regulation when COX defects are caused by mutations or knock-out of *SURF1* gene, coding for COX-specific assembly protein, or when COX deficiency results from mtDNA 9205delTA microdeletion that affects genes encoding mitochondrially synthesized COX3 and ATP6 subunits. In addition, the study also focused on possible specific interactions of COX enzyme with respiratory chain flavoprotein dehydrogenases. The thesis is based on longstanding research of COX pathologies in the Department of bioenergetics at the Institute of Physiology CAS, in collaboration with other research teams from the 1st Faculty of Medicine, CU in Prague, the Children's Memorial

Health Institute in Warsaw, Poland, the Molecular Neurogenetics Unit of Institute of Neurology ”C. Besta” in Milano or MRC in Cambridge, UK.

Specific aims of the thesis were:

(*i*) To compare the impact of *SURF1* gene mutations on the expression of the OXPHOS genes and other pro-mitochondrial genes at the transcriptional and protein levels in *SURF1* patients' fibroblast cell lines.

(*ii*) To investigate the isolated COX defect due to human *SURF1* gene mutations with respect of the mechanism of COX assembly process and ability of COX to interact into respiratory chain supercomplexes.

(*iii*) To characterize tissue/cell specificity of COX defect in *SURF1* knock-out mouse and to find out interspecies differences in the impaired process of COX biogenesis, from early assembly intermediates to formation of COX supercomplexes in fibroblasts from *SURF1* knock-out mouse and *SURF1* patients.

(*iv*) To elucidate mechanism responsible for different severity of clinical manifestations caused by 9205delTA microdeletion of mtDNA, which impairs the expression of COX3 and ATP6 subunits and thus structure and function of COX and ATP synthase (CV).

(*v*) To investigate possible non-canonical supercomplex formations as interactions of COX enzyme with respiratory chain flavoprotein dehydrogenases CII (SDH) or mGPDH.

3. SUMMARY OF THE RESULTS

The thesis consists of five publications. The first two publications are focused on detailed description of isolated COX deficiency in patients' cells with different mutations of *SURF1* gene, from whole genome transcript levels determination and the correlation with adaptive changes in protein content of other RC complexes, to the disturbed COX assembly pathway due to SURF1 protein absence, respectively. They further describe species-specific differences in COX assembly process in humans and mice and analyze how the COX biogenetic pathway is influenced by human *SURF1* gene mutation or knock-out of mouse *SURF1* gene. Finally, they demonstrate differences in kinetics of COX holoenzyme and COX supercomplexes formation that underlie interspecies differences in SURF1 absence.

The third paper addresses COX deficiency due to mtDNA 9205delTA mutation. With the help of cybrid cell lines it shows that pathogenic mechanism originating from altered synthesis of COX3 subunit depends on steep threshold of mutated mtDNA.

The fourth and fifth paper are dedicated to rather unexplored theme concerning the possible interaction of COX with FAD-dependent RC dehydrogenases CII (SDH) and mGPDH into higher structures/supercomplexes in mammalian respiratory chain.

1. Adaptation of respiratory chain biogenesis to cytochrome *c* oxidase deficiency caused by *SURF1* gene mutations. Nikola Kovářová, Alena Čížková - Vrbacká, Petr Pecina, Viktor Stránecký, Ewa Pronicka, Stanislav Kmoch, Josef Houštěk. *Biochim. Biophys. Acta*, 2012, 1822: 1114–1124. IF = 4.882

This study was based on analysis of fibroblast cell lines from 9 patients with different *SURF1* gene mutations that are frequent cause of severe COX defects with clinical manifestation of the Leigh syndrome - a fatal neurodegenerative disease. These mutations lead to loss of a SURF1 assembly protein, which is supposed to participate in an early step of COX assembly process during the association of COX2 subunit with the assembly intermediate S2 composed of COX1-COX4-COX5a subunits. We focused first on adaptive-compensatory changes induced by a decrease of COX level (to 30%) in *SURF1* patient's mitochondria which resulted in up-regulation of OXPHOS complexes I, III and V (130-150%) and accumulation of COX5a subunit.

We have studied expression of genes coding for OXPHOS structural subunits as well as the genes related to mitochondrial biogenesis and functional regulations with respect of changes observed in the protein content of OXPHOS complexes. Whole genome expression profiling revealed a general decrease of transcriptional activity in *SURF1* patient's cells with the most down-regulated transcripts for NDUFA4, NDUFB6 of CI and IF_1 of CV. Only two mRNA for nuclear encoded COX subunits COX7a2 and COX6c corresponded with the decrease of COX complex while no significant up-regulation of the regulatory genes of mitochondrial biogenesis was observed. The compensatory increase in several OXPHOS complexes therefore originated from posttranscriptional changes in later stages of protein biosynthesis.

Then we investigated alteration of COX assembly process as a consequence of *SURF1* mutations and possible differences in supercomplex (SC) formation due to decreased amount of COX. Using 2D electrophoretic analysis we demonstrated that two comigrating COX1 assembly intermediates accumulate in the 85-130 kDa region in COX-deficient *SURF1* patient fibroblasts. The smaller one was the presumed S2 subcomplex consisting of COX1, COX4 and COX5a subunits, the bigger one contained large amount of COX1 subunits associated with other proteins/complexes that might be connected with COX1 biogenesis. Fully assembled and functional COX was preferentially found in I-III_2-IV_1 SC, possibly to take an advantage of

substrate channeling through respiratory chain in otherwise energy-deficient *SURF1* patients´ cells. Consequently, lack of COX in *SURF1* patients further led to accumulation of I-III_2 SC and CIII dimer, as well as disappearance of COX dimer and III_2-IV SC, on the other hand.

2. Tissue- and species-specific differences in cytochrome *c* oxidase assembly induced by *SURF1* defects. Nikola Kovářová, Petr Pecina, Hana Nůsková, Marek Vrbacký, Massimo Zeviani, Tomáš Mráček, Carlo Viscomi, Josef Houštěk. *Biochim. Biophys. Acta*, 2016, 1862(4), 705-715. IF = 4.882

In this study we aimed at distinct biochemical phenotype of COX deficiency in mouse and humans caused by absence of SURF1 protein, an important ancillary factor needed for efficient COX biogenesis. This study was based on collaboration with research group of Prof. Zeviani from Molecular Neurogenetics Unit of Institute of Neurology "C. Besta" in Milano and MRC in Cambridge, who created an unique mouse model of *SURF1* gene knock-out (*SURF1*$^{-/-}$ mouse). *SURF1*$^{-/-}$ knockout in mouse surprisingly results in mild COX deficiency and no neurodegenerative disorder in contrast to patients with *SURF1* gene mutations, who suffer from severe Leigh syndrome. We therefore investigated *SURF1*$^{-/-}$ mouse tissues and fibroblasts in comparison with patient fibroblasts lacking SURF1 protein in a view of impaired COX assembly process and COX ability to incorporate into respiratory supercomplexes (SCs). Our results revealed, that COX activity was considerably decreased in *SURF1* patient fibroblasts compared to *SURF1*$^{-/-}$ mouse tissues/fibroblasts. Using 2D electrophoretic analysis we have found accumulation of abundant COX1 assembly intermediates and low content of fully assembled COX monomer preferentially recruited into I-III_2-IV_1 SCs in *SURF1* patient fibroblasts, as was actually described in the first paper of this thesis. In contrast, *SURF1*$^{-/-}$ mouse tissues/fibroblasts showed lower accumulation of COX1 assembly intermediates, which were markedly variable, milder decrease in COX monomer, which appeared more stable, and very low amount of I-III_2-IV_n SCs. The COX defect was the least pronounced in *SURF1*$^{-/-}$ mouse liver and brain, whereas it was more severe in *SURF1*$^{-/-}$ mouse fibroblasts. We further analyzed kinetics of COX biogenesis in *SURF1* patient and *SURF1*$^{-/-}$ mouse fibroblasts by doxycycline reversible arrest of mitochondrial translation and ^{35}S-labeling of mtDNA encoded proteins. During the gradual recovery after doxycycline inhibition, COX monomer and SCs distributed in rather stable proportion in human control cells while COX monomer markedly decreased and formation of SCs was preferred in *SURF1* patient cells. In *SURF1*$^{+/+}$ and *SURF1*$^{-/-}$ mouse cells, the recovery proceeded mainly to the level of COX monomer and COX SCs formed only temporarily during recovery process. Pulse-chase metabolic labeling showed a higher stability of COX monomer

and faster degradation/depletion of COX assembly intermediates in *SURF1*$^{-/-}$ mouse fibroblasts, whereas gradually decreasing signal of COX monomer was accompanied with persistent abundant COX assembly intermediates in *SURF1* patient cells. Our study thus clearly demonstrated better tolerance of SURF1 protein absence in mouse cells compared to *SURF1* patient cells, where COX biogenesis is much less effective.

3. Alteration of structure and function of ATP synthase and cytochrome *c* oxidase by lack of F_o-a and COX3 subunits caused by mitochondrial DNA 9205delTA mutation. Kateřina Hejzlarová, Vilma Kaplanová, Hana Nůsková, Nikola Kovářová, Pavel Ješina, Zdeněk Drahota, Tomáš Mráček, Sara Seneca and Josef Houštěk. *Biochemical Journal*, 2015, 466: 601–611. IF = 4.396

Cytochrome *c* oxidase (COX) deficiency can be caused also by numerous missense mutations in mtDNA encoded subunits. A different type of pathogenic mechanism is represented by very rare 9205delTA microdeletion of mtDNA, which removes the STOP codon of *MT-ATP6* gene and alters the splicing site for processing of the polycistronic transcript *MT-ATP8/MT-ATP6/MT-COX3*. This mutation was found only in two patients, supposedly harboring homoplasmic mutation, but surprisingly presenting with highly different severity of the diseases. In fibroblast cells of a patient with fatal clinical course, pronounced decrease of ATP production was accompanied with reduced content of COX and altered ATP synthase structure, whereas no significant biochemical changes could be observed in a patient with mild phenotype. The possible reason could be mtDNA heteroplasmy, which was unmasked during the prolonged cultivation of fibroblast cells of the latter patient, where presence of wt mtDNA indicated that negative segregation of 9205delTA mutation occurred. Therefore, to learn more about the pathogenic mechanism of 9205delTA mutation, cybrid cell lines with different heteroplasmy were prepared, ranging from 52% to more than 99% 9205delTA mutation load. In these 9205delTA homoplasmic cybrids, the amount of COX3 subunit was strongly reduced as well as other mtDNA coded subunits COX1 and COX2, whereas nuclear encoded COX subunits were affected less, compared to control wild-type mtDNA homoplasmic cybrid cells. As a consequence, COX biosynthesis was strongly diminished but the structure of the enzyme was not influenced. The content of F_o-a subunit, coded by *MT-ATP6* gene, was also strongly decreased in 9205delTA homoplasmic cybrids, but other ATP synthase subunits were unaffected or even slightly increased. This led to alteration of ATP synthase structure, which assembled into 60 kDa smaller enzyme complex devoid of F_o-a subunit, retaining hydrolytic activity, but unable to synthetize ATP. Decreased amount of COX and non-functional ATP synthase thus prevented the

most of the mitochondrial ATP production. Normal levels of both COX3 and F_o-a subunits were present up to ~ 90% mutation heteroplasmy, whereas above this threshold, the biochemical changes caused by 9205delTA mutation manifested with steep dependence on increasing mutation load. Importantly, the COX activity, ATP synthesis and ADP-stimulated respiration showed the same dependence on 9205delTA mutation load, thus resulting in near-linear relationship among functional parameters and F_o-a and COX3 subunits content. Therefore, different phenotypes observed in the two patients could be explained by the threshold effect of 9205delTA mutation originating from a gene-protein level.

4. High molecular weight forms of mammalian respiratory chain complex II. Nikola Kovářová, Tomáš Mráček, Hana Nůsková, Eliška Holzerová, Marek Vrbacký, Petr Pecina, Kateřina Hejzlarová, Katarína Kľučková, Vojtěch Rohlena, Jiří Neužil, Josef Houštěk. *PLoS ONE*, 2013, 8(8): e71869. IF = 3.234

Mitochondrial respiratory chain complexes are known to interact into higher structures - supercomplexes (SCs), which can be well preserved in mild detergent solubilizates and isolated by native electrophoretic systems. These SCs usually consist of complexes I, III and IV, but involvement of complex II (CII), linking respiratory chain with tricarboxylic acid (TCA) cycle, in SCs structures remains questionable. In this study we found CII associated in higher molecular weight structures (CII_{hmw}), which were enzymatically active and differed in electrophoretic mobility between different tissues (500 - over 1000 kDa) and cultured cells (400 - 670 kDa). CII_{hmw} structures were well resolved only under very mild conditions of isolation, using digitonin solubilisation and clear native electrophoresis. In the presence of n-dodecyl-ß-D-maltoside or Coomassie blue G dye, routinely used in blue native electrophoretic analysis, CII_{hmw} were rather unstable and readily dissociated into CII monomer and individual subunits. The existence of CII_{hmw} led us therefore to search for possible interaction partners of CII. To test the dependence of CII_{hmw} formation on other respiratory chain complexes we analyzed human fibroblasts with different types of OXPHOS defects, ranging from mtDNA devoid cells to isolated OXPHOS defects, including the COX deficient patient fibroblasts with *SURF1* gene mutation. Isolated COX defect would potentially lead to decrease/absence of CII_{hmw} structures, if COX interacts with CII. However, clear native electrophoretic analysis clearly showed presence of CII_{hmw} structures in *SURF1* patients' cells, as well as in other types of isolated OXPHOS defects. We also did not observe structural interactions of CII with other TCA cycle enzymes, but our immunoprecipitation experiments pointed to a specific interaction of CII with ATP synthase, which confirmed CII as a structural component of mitochondrial ATP-sensitive K^+

channel. Thus, our study excluded respiratory chain CII to be a part of respirasome SC, but existence of CII_{hmw} structures indicated, that CII or its subunits may have another role beyond direct involvement in the mitochondrial bioenergetics.

5. ROS generation and multiple forms of mammalian mitochondrial glycerol-3-phosphate dehydrogenase. Tomáš Mráček, Eliška Holzerová, Zdeněk Drahota, Nikola Kovářová, Marek Vrbacký, Pavel Ješina and Josef Houštěk. *Biochim Biophys Acta*, 2014, 1837(1):98-111. IF = 5.353

Mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) belongs to the group of enzymes representing the respiratory chain (RC) but its expression is highly tissue specific. The highest activity of mGPDH is known to be in mammalian mitochondria of brown adipose tissue (BAT) and in this study we used rat BAT to analyze different forms of mGPDH occurring in vivo. Using native electrophoretic systems we found, that mGPDH solubilized by digitonin from BAT mitochondria is present in several homooligomeric forms, most likely as dimer, trimer, tetramer but also in high molecular weight SCs of more than 1000 kDa. mGPDH SCs were labile in the presence Coomassie blue G dye or at increased concentration of detergent digitonin. This suggests, that weak electrostatic interactions are responsible for mGPDH SCs formation, similarly as in case of CII higher structures described in the study above. As respiratory chain complexes CI, CIII and COX interact into SC forms, which may support the electron transport activity of the RC and also stability of interacting enzymes, it was of interest to investigate possible interactions of mGPDH with other RC complexes. However, detailed electrophoretic analysis did not confirm any associations with CI, CIII or COX, although it would make kinetic sense because of sharing of common electron transfer pathway. Moreover, mGPDH SCs were capable of ROS production, which production could be prevented, if mGPDH was bound in RC SCs ensuing more efficient channeling of electrons to COX.

Contributions of dissertant to these publications

All the findings presented in the five studies above are results of teamwork, the following are the concrete contributions of the dissertant:

1. Cultivation of control and SURF1 protein deficient human and mouse skin fibroblast cell lines, isolation of cell membranes and mitochondria from fibroblast cell lines and isolation of mitochondria from different types of rodent tissues.

2. Quantification of OXPHOS complexes using SDS electrophoresis in combination with immunodetection, structural determination of COX subcomplexes and respiratory chain supercomplexes using different native and two-dimensional electrophoretic systems in combination with immunodetection.
3. In-gel activity measurements of OXPHOS complexes.
4. Spectrophotometric activity measurements of OXPHOS complexes.
5. Immunoprecipitation analysis for determination of CII interaction partners in its higher structures.
6. Proteomic analysis of COX subcomplexes and CII higher structures: preparing of samples for mass spectrometry using in-gel digestion of proteins isolated by native electrophoretic systems.
7. Doxycycline treatment and pulse-chase metabolic labeling of mtDNA encoded proteins of control and SURF1 protein deficient human and mouse skin fibroblast cell lines.

4. CONCLUSIONS

Based on the results obtained in studies summarized in the thesis, the following conclusions can be made:

1. Decreased content of COX in *SURF1* patients' fibroblasts was accompanied by the compensatory upregulation of complex I, III and V, which originated from posttranscriptional events, because no specific upregulations of OXPHOS genes or mitochondrial regulatory genes at the level of mRNA expression could be observed.

SURF1 gene mutations lead to disturbed COX assembly process characterized by accumulation of abundant COX1 assembly intermediates - the S2 subcomplex containing COX1-COX4-COX5a subunits, which comigrate with subcomplex(es) containing COX1 and perhaps other proteins, respectively. This may reflect the impaired efficacy of COX1 biogenesis and its delayed interaction with other COX subunits due to SURF1 protein absence.

Decreased amount of fully assembled COX is preferentially recruited into respiratory I-III_2-IV_1 SC in *SURF1* patient cells, which is supposed to stabilize the COX enzyme and enables substrate channeling for more efficient energy production.

COX assembly kinetics was considerably different in *SURF1* patient cells compared to control cells. Newly synthetized COX monomer was unstable, rapidly decreased, and only small amount was bound into I-III_2-IV_1 SC, whereas incomplete COX forms accumulated over the

course of time in *SURF1* patient cells. Control cells, in contrast, gradually established a distribution balance among COX SCs, monomer and subcomplexes.

2. In *SURF1*$^{-/-}$ mouse with knock-out of *SURF1* gene, the amount and activity of COX enzyme was decreased, the decrease was tissue/cell specific, but was not as pronounced as in *SURF1* patient cells. COX1 assembly intermediates accumulated due to SURF1 protein absence and again varied among different tissues and fibroblasts.

An important difference between *SURF1*$^{-/-}$ mouse and *SURF1* patient fibroblasts we uncovered was, that I-III_2-IV_n SCs represented just a small portion of the total COX content and COX monomer remained the dominant form in mouse cells. This pattern was characteristic also for mouse control cells.

Assembly kinetics of COX proceed mainly at the level of COX monomer and COX assembly intermediates over the course of time in *SURF1*$^{-/-}$ mouse cells, newly synthetized COX monomer was more stable and COX assembly intermediates were faster depleted/degraded compared to *SURF1* patient fibroblasts. SURF1 protein thus seems to be more required for effective COX assembly process in humans, than in mouse.

3. Detailed study of cybrid cell lines with different mutation load of 9205delTA mutation in mtDNA affecting synthesis of F_o-a and COX3 subunits explained different severity of pathogenic phenotypes in two patients. The disease manifestation was ascribed to the level of heteroplasmy exceeding the 90% threshold of the mutation that was linked with formation of incomplete ATP synthase unable to synthetize ATP due to the lack of F_o-a subunit and with decreased level of COX3 subunit that led to downregulation of assembled COX enzyme. In these pathogenic conditions caused by threshold effect of 9205delTA mutation, the combined defect of both ATP synthase and COX prevented the most of the mitochondrial energy production.

4. COX interactions with RC flavin dehydrogenases CII (SDH) and mGPDH into higher molecular weight structures were not found/confirmed despite application of several approaches previously utilized to uncover such a type of interactions. These enzymes perhaps occur mainly in their multimeric forms or form SCs with other proteins with the functional relevance beyond the RC, as mitochondrial ATP-sensitive K^+ channel in case of CII.

DISCUSSION

COX is structurally and functionally very complicated protein complex, which correct and exact assembly process depends on many ancillary proteins, among which belongs also the SURF1 protein. Mutations in *SURF1* gene lead to absence of this protein, resulting in severe COX defects manifesting as fatal neurodegenerative Leigh syndrome within several months after the birth. On the contrary, knockout of *SURF1* gene in mouse organism is accompanied by much less pronounced COX defect that does not lead to development of any associated pathology as exemplified by uncompromised lifespan and welfare of these animals.

In analyzed fibroblast cell lines of patients with different *SURF1* gene mutations, main changes were observed at the level of protein expression. Decreased amount of COX holoenzyme to about 30% was accompanied by upregulation of other OXPHOS complexes I, III and V, most likely as a compensatory reaction of *SURF1* patient´s cells, which was not observed in mRNA transcript levels. However, this adaptive upregulations did not rescue disturbed energetic metabolism due to the severe COX defect. It seems, that compensatory upregulations of OXPHOS or other mitochondrial proteins are rather frequent responses of cells/tissues to mitochondrial disorders. For instance, increased amount of COX and CIII was found in fibroblasts of patients with isolated deficiency of ATP synthase due to *TMEM70* gene mutation. As in *SURF1* patient fibroblasts, mRNA transcript levels of respective proteins were not changed [66]. Increased amounts of ANT and PIC (ADP/ATP translocase and inorganic phosphate carrier, respectively) in fibroblasts from ATP synthase deficient patients [67] or accumulated level of CII in ragged-red muscle fibers of MERRF patients [68] may be other examples, how cells/tissues respond to given pathophysiologic state. Nevertheless, the functional relevance of such adaptive changes is mostly questionable.

COX assembly pathway was markedly affected at first steps in *SURF1* patient's cells, S2 assembly intermediate accumulated together with another COX assembly intermediate containing COX1 subunit. It means, that SURF1 protein absence led to disturbed/slowed down biogenesis of COX1 and also impaired subsequent process of COX assembly, from interaction of COX2 subunit with S2 intermediate up to final COX monomer. Interestingly, as a reaction on COX defect, fully assembled COX monomer was preferentially incorporated into I-III_2-IV_1 SC in *SURF1* patient's cells. COX usually interacts into large SCs, but its significant portion occurs in monomeric form in control cells. Because COX is dramatically decreased in *SURF1* patient cells, its preferred incorporation into SC structure could improve COX stabilization and help the cells to cope partly with the energetic deficiency, considering the advantage of direct electron transfer from CI to COX in SCs. In previous study of Pecina

et al. [36] it was shown, that the functional properties of COX were considerably altered in *SURF1* patient's cells. The enzyme seems to have upregulated electron transfer activity, which could be explained by the fact, that major portion of COX associated in SCs participates in electron transfer from substrate to oxygen. On the other hand, impaired proton pumping activity of COX and decreased affinity to oxygen [69] may underline the pathologic phenotype of *SURF1* mutations in patients.

In mouse with *SURF1* gene knockout (*SURF1*$^{-/-}$), tissue specific decrease of COX amount and activity were found and the highest COX defect showed *SURF1*$^{-/-}$ mouse fibroblasts. Compared to *SURF1* patient fibroblasts, the amount of fully assembled COX monomer was higher and formation of I-III$_2$-IV$_1$ SC was less significant. Kinetic analysis of COX monomer assembly and its interaction into SCs revealed marked differences in *SURF1*$^{-/-}$ mouse and *SURF1* patient fibroblasts, newly assembled COX monomer was of higher stability and COX assembly intermediates were faster depleted/degraded in *SURF1*$^{-/-}$ mouse than in *SURF1* patient cells. Contrariwise, more SCs were formed in time, which final amount was higher compared to COX monomer in *SURF1* patient cells. These results showed, that human and mouse fibroblasts react differently on COX defect due to SURF1 protein absence and that *SURF1*$^{-/-}$ mouse cells probably cope better with this conditions. *SURF1*$^{-/-}$ mouse fibroblasts displayed similar pattern of COX functional alterations as *SURF1* patient's fibroblasts, which were less pronounced as expected due to milder COX defect. The most pronounced functional changes were observed also in the COX affinity for oxygen (manuscript in preparation).

As a common consequence of SURF1 defect in human and mouse cells/tissues is the accumulation of COX1 assembly intermediates that characterizes impaired efficiency of COX1 biogenesis and its delayed interaction with other COX subunits. During the COX1 maturation, catalytic components like hemes and Cu_B are incorporated with the help of COX specific assembly proteins. Shy1, yeast homolog of SURF1, was found to form early intermediate with COX1, where both heme *a* and heme a_3 cofactor sites are formed in a stepwise process and presumably also Cu_B site [70,71]. Moreover, Shy1 most likely cooperates in heme *a* transfer and insertion into early COX1 assembly intermediates, because it was found in association with heme *a* synthase COX15 [40]. Based on these findings we can only suppose analogous function of SURF1 in the mammalian cells/tissues, but it has not been experimentally proved yet. As ensures from our results, the COX assembly process seems to proceed faster in mouse than in human fibroblasts. Especially faster assembly, rapid recycling of unincorporated COX subunits and production of higher steady state COX content

in *SURF1*$^{-/-}$ mouse cells may suggest some similar compensatory mechanism as described in yeast Shy1 mutant, which phenotype was partially restored by the suppressor *MSS51* through increased translation of Cox1 subunit [72]. Nevertheless, the decrease in the amount of newly synthetized COX1 subunit was approximately equal in both mouse and human fibroblasts with SURF1 defect without any indication of compensatory upregulation in mouse cells. Based on diversity of yeast and mammalian mitochondrial genome, mammals do not have functional homolog of translation activator Mss51p, which is primarily specific translation factor for yeast *COX1* mRNA, and expression of mammalian COX1 is thus markedly different. This suppression mechanism of COX assembly defect is therefore solely chatacteristic for yeast.

Another cause of investigated COX assembly defect was specific 9205delTA microdeletion in mtDNA, which alters processing of the polycistronic transcript of the genes for ATP8, ATP6 and COX3 subunits. Detailed analysis of different cybrid clones with variable mutation load revealed, that after exceeding of the 90% level of mutated mtDNA, COX and ATP synthase defects manifest with steep dependence on increasing mutation load. In these conditions, reduced amount of COX holoenzyme and synthesis of non-functional ATP synthase led to energetic defect in cells that resulted in severe clinical manifestations in described patient with the mutation. Thus, such threshold effect with resulting fast biochemical changes means, that a few % increase in mutation load can cause phenotypic changes from benign clinical course [73] to fatal disorder [74]. It seems, that primarily more critical is COX deficiency for the disease progression, because low content of COX dramatically decreases the overall proton translocating activity of the respiratory chain in homoplasmic 9205delTA cybrids and thus would prevent/limit the energetic function of ATP synthase, even if it was fully functional.

COX occurs often in its SC forms in the IMM. Interactions of COX with FAD-dependent dehydrogenases of the respiratory chain, glycerol-3-phosphate dehydrogenase (mGPDH) and succinate-CoQ oxidoreductase (CII, SDH), however, were not detected in this thesis. The initial reasoning was, that interactions of CII/mGPDH with CIII and COX might be functionally relevant, because such SCs could ensure more efficient electron transfer from given dehydrogenase up to COX and prevent from possible electron leak and ROS formation considering, that both CII and mGPDH are important sites of electron leak and ROS generation [75,76]. We have succeeded to uncover large supramolecular structures of CII and of mGPDH, but the only interaction with other OXPHOS complexes we have found was that of CII with CV - ATP synthase, supporting previous studies suggesting that both of these

complexes exist in a SC comprising the mitochondrial ATP-sensitive K^+ channel (mK_{ATP}), where CII functions as a regulatory component [77,78]. The mK_{ATP} is a central component of ischemic preconditioning mediated protection against ischemia reperfusion injury. mK_{ATP} channel openers, such as specific inhibitors of CII, can mimic ischemic preconditioning and are therefore cardioprotective. Thus, CII may be a potential component for therapeutic target in ischemia reperfusion injury. mGPDH interaction into higher supramolecular structures characterized in our study disproved mGPDH participation in respirasome SC, but it may suggests interaction with some other mitochondrial proteins and thus another role of mGPDH beyond the entire energetic metabolism.

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