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The Consequences of a Mild Respiratory Chain Deficiency on Substrate Competitive Oxidation in Human Mitochondria

Vanna Geromel, Béatrice Parfait, Jürgen-Christoph von Kleist-Retzow, Dominique Chretien, Arnold Munnich, Agnès Rötig, and Pierre Rustin[1]

Unité de Recherches sur les Handicaps Génétique de l'Enfant, INSERM U393, Hôpital des Enfants-Malades, Paris, France

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The competition between the respiratory substrates to gain access simultaneously to the respiratory chain depends on the dehydrogenase activity, the mitochondrial ubiquinone pool, and the oxidizing activity of the cytochrome segment. By studying the co-oxidation of NADH and succinate by control human liver homogenates, we found that a change in the balance between respiratory chain complex activities may affect significantly the ability of the mitochondria to oxidize one or the other substrate. Accordingly, in the particular case of a patient presenting with a partial complex I and IV deficiency, we observed a strongly reduced ability to oxidize NADH in the presence of succinate. It therefore appeared that even a slight imbalance between respiratory chain enzyme activities may result in a full blockade of a given substrate oxidation.

The mechanism by which moderate defects of the respiratory chain (RC)[1] lead to severe clinical phenotypes raises intriguing questions. High residual activities associated with imbalance between RC complex activities have often been reported in patients with RC deficiency (1-4). In patients harboring mtDNA mutation high residual activities can reflect the coexistence of mutant and wild-type mtDNA (heteroplasmy). However, while heteroplasmy possibly explains the partial character of a defect, it does hardly explain how a mild biochemical defect can result in a severe clinical phenotype. A causative relationship with heteroplasmy can also be disregarded as a general mechanism, since similarly high residual activities have been reported in cases of defects affecting nuclearly encoded RC protein (5,6).

The consequences of a defect in one enzyme of a given metabolic pathway is currently considered to depend upon the amount of control exerted by that enzyme step on the overall pathway flux. Numerous works have been devoted to the determination of control coefficients along the RC (7,8), but although some of these studies have dealt with pathological situations (9,10), no clue has emerged to understand the association of mild biochemical defects to severe clinical phenotypes.

The examination of *in situ* conditions of mitochondrial functioning may help to generate working hypotheses. In particular, one has to bear in mind that mitochondria have to cope simultaneously with a variety of respiratory substrates to meet the cell metabolic demand. The ability of mitochondria to oxidize simultaneously these substrates relies on a controlled balance between the activities of the different complexes inside the RC, particularly between the different dehydrogenases. Accordingly, while the number of mitochondria varies between tissues and according to the cell metabolic demand (11), the balance between the RC complex activities appears quite conserved between tissues in both animal (12) and man (13). As a practical consequence, the analysis of RC activity ratios has revealed as a powerful mean to characterize abnormal RC in patients (14).

Here, we show that an imbalance between the RC dehydrogenase activities can result in the full blockade of specific substrate oxidation.

PATIENT AND METHODS

Patient. A girl was born at term to non-consanguineous parent. She was referred at age 1 month, presenting with a severe convulsive

[1] To whom correspondence should be addressed at INSERM U393, Tour Lavoisier, Hôpital des Enfants-Malades, 149, rue de Sèvres, 75743 Paris Cedex 15, France. Fax: (33) 01 47 34 85 14. E-mail: rustin@necker.fr.

Abbreviations: BSA, bovine serum albumin; CI-CV, the various complexes of the respiratory chain; DUQ, decylubiquinone; GCCR, glycerol-3-phosphate cytochrome *c* reductase; mt, mitochondria; RC, respiratory chain; SCCR, succinate cytochrome *c* reductase; SDH, succinate dehydrogenase.

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TABLE 1

Enzyme Investigations in Frozen Liver Homogenate and Cultured Skin Fibroblasts from Patient and Controls

	Liver homogenate	
	Patient	Controls (n=17)
Activities (nmol acceptor or donor/min/mg protein)		
Complex I	23	11-33 (23)[a]
Complex II	267	93-205 (145)
Complex III	268	100-253 (173)
Complex IV	192	87-291 (189)
Complex V	199	38-119 (86)
Complex I+III	72	26-86 (56)
Complex II+III	128	38-100 (74)
Activity ratios		
Complex II/I	**11.6**	7.1 ± 0.8[b]
Complex IV/V	**1.0**	2.3 ± 0.3
Complex IV/III	**0.7**	1.3 ± 0.2
Complex IV/II	**0.7**	1.5 ± 0.3
Complex IV/I	8.7	8.8 ± 1.2
Complex III/II	1.0	1.2 ± 0.2
Complex III/I	**11.7**	7.9 ± 1.0
Complex III/complex II+III	2.1	2.1 ± 0.3
Complex II/complex II+III	2.0	1.7 ± 0.3

Note. Abnormal respiratory chain enzyme activity ratios are indicated in bold.

[a] Mean value.

[b] Mean value ± 1 SD.

encephalopathy and psychomotor retardation. Hyperlactatemia (4.38 mmol/l; normal 0.63-2.44) with elevated lactate/pyruvate (L/P) molar ratio (45; normal <20) were noticed and suggested possible mitochondrial RC deficiency. Enzyme studies indicated a low activity of CI and CIV relative to the activities of other components of the RC in both liver homogenate and cultured skin fibroblasts.

Methods. Liver tissues (10-20 mg) were homogenized by 5 strokes in a ground glass potter in 50 μl of a ice-cooled medium consisting of 0.25 M sucrose, 40 mM KCl, 2 mM EGTA, 1 mg/ml BSA and 20 mM Tris-HCl (pH 7.2) (15). The homogenate was centrifuged at 2000 *g* for 3 min and measurements carried out on supernatant samples. Spectrophotometric measurements of cytochrome *c* oxidase (CIV; EC 1.9.3.1), malonate-sensitive succinate cytochrome *c* reductase (CII+III), rotenone-sensitive NADH cytochrome *c* reductase activity (CI+III), malonate-sensitive succinate-decylubiquinone-dichlorophenol indophenol (DCPIP) reductase (CII; EC 1.3.5.1), rotenone-sensitive NADH decylubiquinone reductase (CI; EC 1.6.5.3), antimycin-sensitive ubiquinol-cytochrome *c* reductase (CIII; EC 1.10.2.2), oligomycin-sensitive ATPase activity (CV; EC 3.6.1.34) were measured as previously described (15, 16). Polarographic studies were carried out at 37°C in a thermostated 250 μl-cell, fitted with a Clark electrode (Hansatech), and containing 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 1 mg/ml BSA and 10 mM KH_2PO_4 (pH 7.4) (medium **C**) (15). The redox state of pyridine nucleotide was fluorimetrically monitored using a Perkin-Elmer fluorimeter (LS 50 B) at 37°C in a thermostated, magnetically-stirred, quartz cuvette (1×1 cm) containing 2 ml of a medium **C** (17).

RESULTS

The measurement of RC activities in patient liver homogenate revealed increased activities for several RC complexes and, by comparison, a low activity of CI and of CIV, resulting in the abnormality of the activity ratios involving CI or CIV (Table I). Noticeably, residual CI and CIV activities remained well within the control ranges. With the aim to ultimately analyse the potential consequences of an imbalance between RC complex activities (CII/CI) on the ability of the RC to use respiratory substrate (NADH), we next studied the co-oxidation of NADH and succinate.

In human liver homogenate, NADH and succinate oxidations were found sensitive (more than 97%) to rotenone, a specific CI inhibitor, and malonate, a competitive inhibitor of the SDH, respectively (not shown). This ruled out any significant contribution of plasma membrane NADH dehydrogenase to NADH oxidation and provided tools to further discriminate between both types of substrate oxidation during co-oxidation studies.

In the next experiments, NADH and succinate were sequentially added to control liver homogenate and the resulting oxidizing activities were analyzed by polarography and fluorimetry (Fig. 1). The initial addition of NADH triggered an active oxygen uptake that was accompanied by a stoechiometric decrease of the NADH fluorescence (Fig. 1A). As shown in Fig. 1B, the addition of succinate in the presence of NADH caused an increase of oxygen consumption (about 70%), while causing a significant decrease (about 40%) in the rate of NADH oxidation. After a subsequent addition of malonate, oxygen uptake was reduced, while NADH oxida-

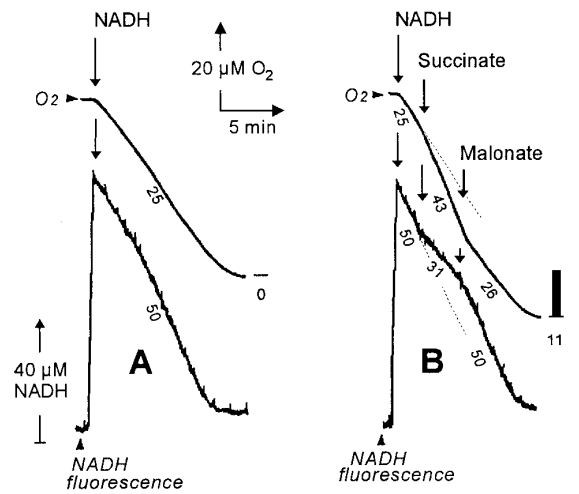

FIG. 1. The competitive mitochondrial oxidation of NADH and succinate in human liver homogenate. (**A**) Oxidation of a limiting amount of NADH (80 μM) simultaneously studied by polarography and fluorimetry. (**B**) Effect of succinate (10 mM) and malonate (10 mM) on NADH oxidation (80 μM). Numbers along the traces are nmol/min/mg protein; the dark bars on the right of the polarographic traces represent the total amount of succinate oxidized during each experiment. Experimental conditions as described under Patient and Methods.

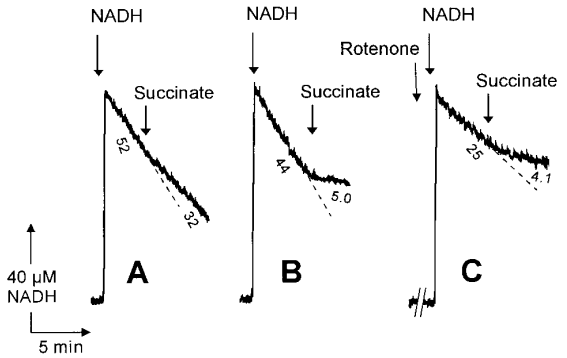

FIG. 2. The effect of a partial respiratory chain defect on substrate competitive oxidation in human liver homogenate. Fluorimetric determination of the effect of succinate on NADH oxidation by control (**A**), patient (**B**) or partially rotenone-inhibited control liver homogenates (**C**). Numbers along the traces are nmol NADH oxidized/min/mg protein. NADH, 80 μM; succinate, 10 mM; rotenone, 30 nM. Experimental conditions as in Fig. 1.

tion resumed to the rate measured before succinate addition. The above experiment showed that, in control liver homogenate, succinate only partially inhibited the NADH oxidation and that this inhibition could be released by inhibiting SDH by malonate.

We next studied the co-oxidation of NADH and succinate in patient liver homogenate (Fig. 2). As it can be inferred from table I, NADH oxidation by patient liver homogenate was not significantly reduced as compared to control (Fig. 2A and B). However, upon succinate addition, the oxidation of NADH was 85% decreased and reached very low value. In the last experiment, NADH oxidation by control liver homogenate was 50% inhibited by an initial addition of rotenone (Fig. 2C). Under these conditions, NADH oxidation could be 85% inhibited by a subsequent succinate addition, mimicking the situation encountered using patient liver homogenate (Fig. 2B).

DISCUSSION

The above results deal with the potential consequences of a partial deficiency of a RC complex on the oxidizing activities of the mitochondria.

Briefly, the RC can be depicted as constituted of three segments, (i) the various dehydrogenases, which feed electrons to the chain, (ii) the ubiquinone pool, which collect the electrons from all the dehydrogenases and (iii) the cytochrome segment, which mediates the reduction of molecular oxygen by electrons. To these, one should add the ATPase, known to indirectly regulate the electron flow by controlling the proton gradient and the membrane potential (18). To collect the electrons from the various dehydrogenases confers on the ubiquinone pool a favored position in the regulation of the electron flew arizing from the various dehydrogenases. Both the redox status and the amount of ubiquinone in the mitochondrial inner membrane are known to differently affect the ability of the dehydrogenases to feed electrons to the RC (19). Thus the SDH is activated by reduced quinones, while the activity of dehydrogenases feeding electrons to CI (malate, pyruvate, α-ketoglutarate dehydrogenases, etc) tends to rapidly slow down with the reduction of the ubiquinone pool (18). The competition between substrate is therefore tightly controlled by the redox status of the ubiquinone pool (19).

Although performed with only two substrates, the results reported in our study show that the consequences of a partial RC defect may be considerably enhanced due to the substrate competition to gain access to the RC. A higher complexity level can be anticipated to happen *in vivo*, in particular the competition may be amplified due to the multiplicity of substrates to be simultaneously oxidized (fatty acids, glycerol-3-phosphate, etc.). The resulting metabolic blockade might then worsen the clinical phenotype predictable from a mild RC defect.

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