Decreased affinity for oxygen of cytochrome c oxidase in Leigh syndrome caused by SURF1 mutations

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ABSTRACT

Mutations in \textit{SURF1} gene prevent synthesis of cytochrome \textit{c} oxidase (COX) - specific assembly protein and result in a fatal neurological disorder, Leigh syndrome. Because this severe COX deficiency presents with hardly detectable changes of cellular respiratory rates at normoxic conditions, we analyzed the respiratory response to low oxygen in cultured fibroblasts harboring \textit{SURF1} mutations using high-resolution respirometry. The oxygen kinetics was quantified by the $p_{50}$ (the pO$_2$ at half-maximal respiration rate) in intact coupled cells and in digitonin-permeabilized uncoupled cells. In both cases, the $p_{50}$ in patients was 2.1- and 3.3-fold elevated, respectively, indicating decreased affinity of COX to oxygen. These results suggest that at physiologically low intracellular pO$_2$ the depressed oxygen affinity may in vivo lead to limitations of respiration resulting in impaired energy provision in Leigh syndrome patients.

KEYWORDS: Cytochrome \textit{c} oxidase, Leigh syndrome, \textit{SURF1} mutations, oxygen kinetics, mitochondrial disease
INTRODUCTION

Oxygen is supplied to tissues through the respiratory cascade. The transport of O$_2$ through the respiratory tract and cardiovascular system is characterized by a drop of the oxygen partial pressure (pO$_2$) from 20 kPa in the inspired air to low intracellular pO$_2$ (22). Under physiological conditions, therefore, mitochondria operate at pO$_2$ as low as 0.3 kPa in some tissues (9). Nevertheless, mitochondrial and cellular respiration in health and disease is rarely studied under physiological low oxygen conditions, and the oxygen affinity of COX is frequently assumed to prevent any oxygen limitations of respiration under normoxia.

Mammalian COX is composed of 13 subunits; mitochondrial DNA (mtDNA) genes encode the 3 largest subunits forming the catalytic core of the enzyme, the other 10 subunits are encoded by the nuclear genome (27). In addition, numerous nucleus-encoded factors are required for efficient assembly and maintenance of the COX holoenzyme that are similar in yeast and humans (11, 18). Mutations in genes encoding these COX assembly factors are a frequent cause of COX deficiencies, in fact they are much more common than mutations in genes encoding the COX subunits themselves (25). One of these assembly proteins is encoded by the $SURF1$ gene. Two groups identified mutations in this gene to be responsible for the autosomal recessive form of Leigh syndrome (LS$^{COX}$) (29, 33). Leigh syndrome, a subacute necrotizing encephalomyopathy, is a progressive neurodegenerative disease. The severe symptoms usually have onset within the first year of life and are characterized by general psychomotor retardation and bilaterally symmetrical lesions in the basal ganglia region. An increased level of lactate in both blood and cerebrospinal fluid is observed. The disease is fatal in vast majority of cases, the patients usually die before five years of age (21). Leigh syndrome is the most common form of COX disorders and one of the most frequently occurring respiratory chain defects in infancy and childhood (23). To date, more than 30 different pathogenic mutations have been described in $SURF1$ (16). Most of them are
nonsense mutations inducing the formation of a premature stop codon; missense and splicing-site mutations are less common. It is generally assumed that the severe isolated COX defect in patients harboring SURF1 mutations results from impaired assembly of the complex (3, 28). In our previous study, we further demonstrated that there exist several functional forms of COX in mitochondria of LS\textsuperscript{COX} cells that differ in subunit composition, electron-transport and proton-pumping properties (15).

As all previous studies with LS\textsuperscript{COX} cells were performed at much higher pO\textsubscript{2} than is actually present in cells in vivo, we focused on the low oxygen levels in our respirometric investigation of the functional impact of SURF1 mutations. We analyzed the oxygen kinetics of COX in fibroblasts from five Leigh syndrome patients by high-resolution respirometry. While respiration at high oxygen levels was indistinguishable from controls in these patient cells, we observed a highly significant decrease of COX oxygen affinity in fibroblasts from all patients studied. These findings are discussed in the context of cell energetics in these pathological states.
METHODS

Patients. Fibroblast cultures of Leigh syndrome patients with isolated COX deficiency caused by SURF1 mutations were provided by Department of Pediatrics, Prague (patients 1 and 2), and Department of Metabolic Diseases, Warsaw (patients 3-5). The SURF1 mutations of fibroblast cultures from five patients are presented in Table 1, all patients harbor premature stop codons, which prevent synthesis of the Surf1 protein. Five different fibroblast cultures without mitochondrial disorder served as controls. The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committee of Medical Ethics. Informed consent was obtained from the parents of all patients.

Cell culture. Skin fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10 % fetal calf serum, 20 mM HEPES pH 7.5, 0.2 % NaHCO₃ and gentamycin (2 mg/100 ml) at 37 °C and 5 % CO₂ in air. Near-confluent cultures were harvested using 0.05 % trypsin and 0.02 % EDTA. Detached cells were diluted in ice-cold culture medium, sedimented by centrifugation (600 g) and washed twice in ice-cold phosphate-buffered saline (140 mM NaCl, 5.4 mM KCl, 8 mM Na₂HPO₄.12 H₂O, 1.4 mM KH₂PO₄, pH 7.2 at 25 °C).

High-resolution respirometry and oxygen kinetics. The Oxygraph-2k (Oroboros, Austria) was used for measurements of oxygen consumption. This instrument provides sufficient sensitivity and time resolution for the analysis of the oxygen kinetics of mitochondrial and cellular respiration (5). Prior to all measurements, the chambers were sterilized with 70 % ethanol for 20 minutes. For calibration of the oxygen sensor, a constant signal was obtained in the opened chamber with medium in equilibrium with the air in the gas phase. Internal zero oxygen calibration was performed using the Datlab software (Oroboros,
Austria) as described (10). All measurements were performed at 30 °C in 2 ml chamber volume.

For measurements with intact fibroblasts, an extracellular TD-buffer (32) was used containing 137 mM NaCl, 5 mM KCl, 0.7 mM sodium phosphate, 25 mM Tris-HCl (pH 7.4). A mitochondrial KCl medium (13) containing 80 mM KCl, 10 mM Tris-HCl, 3 mM MgCl₂, 1 mM EDTA, 5 mM potassium phosphate, (pH 7.4) was used for experiments when the cell membrane of the fibroblasts was permeabilized with digitonin (0.1 g/g protein). In this case, 10 mM succinate was added as a substrate and 1 μM carbonylcyanide p-trifluoromethoxophenylhydrazone (FCCP) was used to uncouple and maximally stimulate respiration (state 3u). The volume-specific rate of oxygen consumption (oxygen flux) was calculated as the negative slope of oxygen concentration recorded at 2 s time intervals. The signal was deconvoluted with the exponential time constant of the oxygen sensor (3 to 5 s). Cellular oxygen flux ($J_{O_2}$) was corrected for instrumental background, which is a linear function of experimental $pO_2$ and results from oxygen consumption by the sensor and oxygen back-diffusion from low capacity oxygen reservoirs. The $p_{50}$ is the partial O₂ pressure, at which the cellular respiratory rate is half-maximal. This parameter was obtained from a hyperbolic function $J_{O_2} = (J_{max.} \cdot pO_2)/(p_{50} + pO_2)$ fitted over the low oxygen range of 0 to 1.1 kPa. All calculations were performed using routine functions of the Datlab software (10).

Protein determination. The protein content was measured in 10 μl and 20 μl aliquots withdrawn from the oxygraph chamber by the method of Bradford (2) using bovine serum albumin as a standard. Samples were sonicated for 20 s prior to protein determination.

Statistics. Data are presented as mean values ± standard deviation from several measurements of each individual cell culture. The statistical significance between control and
patient cells was evaluated using standard t-test and Kruskal-Wallis One-Way ANOVA on Ranks, and Tukey-Kramer Multiple-Comparison Test using freeware version of NCSS software.
RESULTS

The $p_{50}$ was evaluated in two experimental settings – respiration of intact coupled cells with endogenous substrates (controls versus patients P1, P2, P3, and P4), and unrestricted oxidation of exogenous succinate in digitonin-permeabilized cells after FCCP uncoupling (controls versus patients P1, P3, P4 and P5). All these patients presented with typical biochemical phenotype of low COX activity (15, 17).

Typical experimental records of the simultaneous decrease of oxygen concentration and flux are shown in Fig. 1A and 1B for a control and patient (P1) fibroblast culture, respectively. Oxygen flux and the corresponding hyperbolic fits are plotted as a function of oxygen concentration in Fig. 1C. In intact cells the $p_{50}$ of control fibroblasts ranged from 0.03 to 0.05 kPa (0.039 ± 0.010), while in patient cells it increased 2.1-fold to the range from 0.08 to 0.10 kPa (0.082 ± 0.015) (Fig. 2A). No statistically significant differences were found between the average $p_{50}$ of fibroblast cultures of individual patients (not shown). The $p_{50}$ of cells for each patient (P1 - P4), however, was significantly higher compared to controls (Fig. 2A).

Under the conditions of uncoupled state 3 respiration with succinate, the $p_{50}$ of control fibroblasts ranged from 0.020 to 0.042 kPa (0.032 ± 0.010), while in patient cells it increased 3.3-fold versus controls to the range from 0.09 to 0.17 kPa (0.104 ± 0.033) (Fig. 2B). As in intact cells, no statistically significant differences were found between the average $p_{50}$ of fibroblast cultures of individual patients (not shown). The $p_{50}$ of cells for each patient (P1, P3, P4), however, was significantly higher compared to controls (Fig. 2B).

In contrast to $p_{50}$, no statistical differences were found in respiratory rates at kinetic oxygen saturation normalized on protein content ($J_{\text{max}}$) between control and patient cells, in agreement with our previous study (15). In intact control cells, $J_{\text{max}}$ ranged from 42.8 to 99.6 pmol O$_2$·s$^{-1}$·mg$^{-1}$ (mean 60.5 ± 17.9), in patient fibroblasts it ranged from 55.0 to 118.6 pmol
O$_2$·s$^{-1}$·mg$^{-1}$ (mean 78.8 ± 25.4). In state 3u, $J_{\text{max}}$ increased to the range from 60.2 to 215.5 pmol O$_2$·s$^{-1}$·mg$^{-1}$ (mean 108.1 ± 44.5) in controls, compared to 68.1 to 146.0 pmol O$_2$·s$^{-1}$·mg$^{-1}$ (mean 95.0 ± 24.6) in patient fibroblasts.
DISCUSSION

The present study aims at resolving the apparent paradox that fatal \textit{SURF1} mutations associated with isolated COX deficiency present with hardly detectable changes of cellular respiratory rates. We analyzed fibroblasts of five patients harboring mutations of the \textit{SURF1} gene, which prevent synthesis of the Surf1 protein and are responsible for a failure to assemble individual COX subunits into a fully functional enzyme complex in the inner mitochondrial membrane. Leigh syndrome caused by the dysfunction of Surf1 protein is the most common clinical presentation of COX deficiency in childhood (24). Defect of COX activity appears to be the only mitochondrial abnormality in these patients and it is expressed in all tissues including skin fibroblasts. As both the subunit composition of partially assembled complexes (3, 15) and the relative COX capacity are changed in patient cells, one can suspect that oxygen affinity of such COX complexes might be also altered. A decrease of oxygen affinity could then have negative consequences on the oxygen availability for mitochondrial respiration.

We analyzed the COX oxygen kinetics by high-resolution respirometry (10). This method yields the $p_{50}$ parameter, a measure of the sensitivity of cellular respiration to oxygen availability. Such a “macroscopic” approach is advantageous for several reasons: the method is relatively simple compared to e.g. flow-flash kinetic approach which can yield microscopic oxygen binding constants (31). The $p_{50}$ can be used to assess the oxygen dependence of respiration in mitochondria and even small cells with relatively low aerobic activity such as fibroblasts (12) and human umbilical vein endothelial cells (26), where intracellular oxygen gradients are small, and the $p_{50}$ of cellular respiration is close to the $p_{50}$ of isolated mitochondria. Possible changes of intracellular diffusion gradients to mitochondria in pathological cells, therefore, can be ignored in these cells, but might be considerable in more active and larger cells (6). This is supported by our present study, where $p_{50}$ values of control
fibroblasts are similar to $p_{50}$ of mitochondria isolated from rat heart (7). The $p_{50}$, therefore, can be conveniently analyzed in whole cells under conditions of intact mitochondrial membranes, where COX function is integrated in the respiratory chain. Results of such analyses might thus be more relevant in the context of mitochondrial and cellular physiology.

Our results clearly show that $p_{50}$ is increased in patient cells compared to controls. Basically, two reasons for the $p_{50}$ increase can be considered - the $p_{50}$ increase may be due to a decrease of the COX excess capacity, or an increased apparent Michaelis-Menten constant, $K_{m}'$, of COX for oxygen. A lower excess capacity of COX relative to the flux through the respiratory chain yields a corresponding increase of COX turnover, which then causes the $p_{50}$ of oxygen flux through the respiratory chain to increase (8). In order to find out whether the observed increase of $p_{50}$ in patient cells is due to a decrease of COX capacity, we plotted the $p_{50}$ values versus the specific oxygen flux normalized on protein content (Fig. 3). The $p_{50}$ increased as a function of oxygen consumption of intact cells respiring on endogenous substrates (i.e. as COX turnover increased). In patient cells, $p_{50}$ values increased without a corresponding increase of oxygen flux, indicating that the difference in $p_{50}$ cannot be explained by the change of protein-specific flux observed in control and patient cells (Fig. 3A). Although the dependence of $p_{50}$ on oxygen flux is more complex under uncoupled conditions (26), an analogous picture appeared when analyzing $p_{50}$ measurements in FCCP-treated cells (Fig. 3B). These data, therefore, provide strong evidence that the $p_{50}$ increase in patient fibroblasts was due to changes of COX affinity for oxygen.

The most important aspects of the present study are the consequences of a decreased affinity for oxygen on the energetics of the patient cells in vivo. Our previous study indicates that the presence of incomplete COX assemblies with upregulated electron-transport properties might serve as a kind of compensatory mechanism for the lower content of COX subunit I, allowing for near-normal oxygen flux through the respiratory chain under
conditions of high \( pO_2 \) (15). The present data suggest that the situation might be completely different in vivo at low physiological oxygen levels, when oxygen flux would be significantly depressed in patient tissues. Although the \( SURF1 \) genetic defect is present in all cells, our results indicate that it would more pronouncedly manifest in tissues where energy demands are high relative to the cells’ capacity to generate energy. This applies to the central nervous system (CNS), the principal site of pathology in Leigh syndrome. The CNS has very small energy reserves (for review see (1)), and oxygen tensions well below 1 kPa were reported in brain of experimental animals. The rate of oxygen utilization, in particular in neurons, is high and it is expected that rapid consumption by the mitochondria creates around them a "well" in which \( pO_2 \) is lower than at other cellular sites (4). This indicates that cytochrome \( c \) oxidase in \( LS^{COX} \) patient nerve cells would not be kinetically saturated by oxygen. Based on our measurements, for example, at \( pO_2 \) of 0.3 kPa the mitochondrial respiration in patients would be limited to 70 % compared to 90 % saturation in controls. Such oxygen limitation of mitochondrial respiration in patient cells would cause impairment of oxidative energy production at normoxic conditions and especially under hypoxia. This hypothesis is supported by the fact that clinical symptoms in patients with Leigh syndrome significantly worsen during respiratory infections (19, 20), especially when the oxygen supply to tissues is decreased (30).

In addition, a recent study shows that the decrease of COX affinity for oxygen caused by nitric oxide resulted in decreased levels of hypoxia induced factor 1-alpha and thus lower expression of “hypoxic genes” (14). If an analogous situation occurs when COX oxygen affinity is decreased due to \( SURF1 \) mutations, then the patients would not only have impaired oxidative energy production due to oxygen limitation, but would also suffer from impairment of glycolytic energy production due to lower expression of hypoxic genes.
Taken together, the observed phenomenon of increased $p_{50}$ in $L_S^{COX}$ fibroblasts could be one of the key mechanisms that translate the COX assembly defect triggered by $SURF1$ mutation to deleterious changes of cell energetics that finally result in fatal symptoms of Leigh syndrome. In general, experimental and clinical investigations of cells and isolated mitochondria should be extended into the low physiological oxygen range, including evaluation of COX oxygen kinetics, to study the etiopathogenic mechanisms of COX deficiencies.
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REFERENCES


LEGEND TO FIGURES

Fig. 1. Aerobic/anoxic transition of fibroblast respiration. The figure shows a typical record of simultaneous decrease of oxygen partial pressure (right y-axis; full line) and volume specific oxygen flux (left y-axis; ○) during the aerobic/anoxic transition in intact control (A) and patient fibroblasts (B) respiring on endogenous substrates. The parameters of the hyperbolic fit ($J_{\text{max}}$ and $p_{50}$) are shown in the inset. (C) Oxygen flux (individual data points) and corresponding hyperbolic fits calculated by DatLab2 (full line) are plotted as a function of oxygen pressure using data from (A) and (B); ○ control, Δ patient.

Fig. 2. Mean $p_{50}$ of respiration in intact cells (A) and of respiration at state 3u (B).

Data in (A) are displayed for all controls (C, n = 14), for all patients (P, n = 17), and for individual patients P1 (n = 3), P2 (n = 2), P3 (n = 7), and P4 (n = 5). Data in (B) are displayed for all controls (C, n = 17), for all patients (P, n = 23), and for individual patients P1 (n = 8), P3 (n = 7), P4 (n = 7), and P5 (n = 1). Mean values are shown inside the data boxes, error bars indicate ± SD; * $P<0.01$, † $P<0.05$.

Fig. 3. The relation of $p_{50}$ and protein-specific oxygen flux. $p_{50}$ values (kPa) from individual measurements were plotted as a function of protein-specific oxygen flux ($J_{\text{max}}$). (A) endogenous respiration: ▲ control fibroblasts, ○ P1, ◆ P2, ■ P3, * P4. (B) uncoupled respiration: ▲ control fibroblasts, ○ P1, ■ P3, * P4, ◆ P5
### Table 1. *SURF1* mutations in fibroblast cultures of patients P1 to P5

<table>
<thead>
<tr>
<th>Patient</th>
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<td>Frameshift/frameshift:stop codon 870-872</td>
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<tr>
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<td>Arg230&gt;stop/Arg230&gt;stop</td>
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<tr>
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<td>9/-</td>
<td>845 delCT/?</td>
<td>Frameshift: stop codon 870-872/?</td>
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Figure 2

A

B