RESTING OXYGEN CONSUMPTION AND IN VIVO ADP ARE INCREASED IN MITOCHONDRIAL MYOPATHY DUE TO COMPLEX I DEFICIENCY

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Submitted

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Abstract

Patients with isolated Complex I Deficiency (CID) in skeletal muscle mitochondria often present with exercise intolerance as their major clinical symptom. We studied in vivo bioenergetics in three of these patients by measuring oxygen uptake at rest and during maximal exercise, together with forearm ADP concentrations [ADP] at rest. Whole-body oxygen consumption at rest (VO₂rest) was measured with respiratory calorimetry. Maximal oxygen uptake (VO₂max) was measured during maximal exercise on a cycle ergometer. Resting [ADP] was estimated from in vivo ³¹P Magnetic Resonance Spectroscopy measurements of inorganic phosphate, phosphocreatine and ATP content of forearm muscle. VO₂rest was significantly increased in all three patients: 128 ± 14 % (SD) of values in healthy controls. VO₂max in patients was on average 2.8-fold their VO₂rest, and was only 28% of VO₂max in controls. Resting [ADP] in forearm muscle was significantly increased, compared with healthy controls (patients: 26 ± 2 (SD) µM, healthy controls: 9 ± 2 (SD) µM).

We conclude that the increased whole-body oxygen consumption rate at rest reflects increased electron transport through the respiratory chain, driven by a decreased phosphorylation potential. The increased electron transport rate may compensate for the decreased efficiency of oxidative phosphorylation (phosphorylation potential) in these patients.
Chapter 2

Introduction

Defects of mitochondrial oxidative phosphorylation (OXPHOS), are known to be associated with an increasing number of diseases, and can theoretically give rise to any symptom, in any organ or tissue dependent upon mitochondrial energy supply (1). The OXPHOS system is comprised of five enzyme complexes located in the mitochondrial inner membrane: the four enzyme complexes of the respiratory chain (complex I, II, III, and IV), which constitute the chain of electron transport to oxygen, and ATP synthase (F_0F_1-ATPase, complex V).

At three of these enzyme complexes electron transport is coupled to transmembrane proton translocation: complex I, III, and IV. Translocation of protons at these coupling sites generates an electrochemical gradient across the mitochondrial inner membrane, the protonmotive force, which is used for ATP synthesis (2).

Patients with defects in components of the respiratory chain and consequently of OXPHOS are generally thought to have an impaired flow of electrons through the respiratory chain. Consequently, a loss of capacity for proton translocation is assumed, when any of the complexes I, III, and IV of the respiratory chain are affected. Indeed, decreased maximal oxidative rates under optimal assay conditions in isolated mitochondria are used as a diagnostic tool, suggestive for impaired electron flow in mitochondria of these patients.

However, in apparent contrast to these findings in isolated mitochondria, in vivo increased resting oxygen consumption rates at the whole-body level have been reported incidentally in patients with defects in oxidative phosphorylation (3,4,5,6).

In addition, separate studies in heterogeneous groups of patients with mitochondrial myopathies, using in vivo ^31^P Magnetic Resonance Spectroscopy (^31^P-MRS), have shown abnormalities in skeletal muscle high-energy phosphate metabolism including a decreased phosphorylation potential and an elevated resting [ADP] in patients compared with controls (5,7). Increased resting oxygen consumption rates were observed in some of these patients (5).

The present study elaborates on these paradoxical in vivo and in vitro findings in OXPHOS patients. Whole-body oxygen consumption at rest and during maximal exercise was measured in three patients with a documented isolated Complex I Deficiency (CID), presenting with exercise intolerance as their major clinical symptom. Oxygen consumption measurements at rest were combined with estimations of tissue [ADP] and phosphorylation potential from ^31^P-MRS studies in forearm muscle.
Patients and methods

Subjects

Three non related female CID patients, ages 20 to 25, were selected for the study (Table 1). Their weights ranged from 52 to 61 kg. Fat free body mass ranged from 41 to 45 kg.

Table 1. Physical characteristics of CID patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>CID patient</th>
<th>Controls</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
<td>#3</td>
</tr>
<tr>
<td>Age at study (yr)</td>
<td>25</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>52</td>
<td>61</td>
<td>57</td>
</tr>
<tr>
<td>Fat free mass (kg)</td>
<td>41</td>
<td>45</td>
<td>44</td>
</tr>
<tr>
<td>Plasma lactate (mmol/L)</td>
<td>2.3</td>
<td>2.7</td>
<td>4.7</td>
</tr>
<tr>
<td>Blood pyruvate (μmol/L)</td>
<td>132</td>
<td>120</td>
<td>192</td>
</tr>
<tr>
<td>Lactate-to-pyruvate ratio</td>
<td>17.4</td>
<td>22.5</td>
<td>24.5</td>
</tr>
</tbody>
</table>

The patients had very similar clinical histories. They were all born from unrelated parents and suffered from easily fatiguable mild muscle weakness dating back to early childhood. Patient #1 experienced a single stroke-like episode at age 13, which resolved spontaneously after a few hours. None of these patients showed signs of central nervous system involvement. Muscle weakness and fatiguability have remained stable over time in all of them. Exercise intolerance therefore dominated the clinical picture at the time of the study.

Patients #1 and #2 had been prescribed riboflavin and carnitine, but discontinued this therapy because they recalled no benefit from this medication. At the time of the study, patient #3 was on riboflavin and carnitine, even though she did not recall much benefit either. All patients were engaged in social activities, with patient #1 a housewife, and patients #2 and #3 working in service jobs. Mitochondrial DNA abnormalities (pointmutations, i.e. 3243 AG MELAS/PEO, 8344 AG MERRF, 8993 TG NARP, 11778 AG LHON, deletions, duplications, or mtDNA depletion) have not been demonstrated in any of these patients, nor
could a maternal pattern of inheritance be shown. Therefore a mutation in a nuclear encoded subunit of complex I was suspected. Lactate-to-pyruvate ratios in blood were increased and ranged from 17.1 to 24.5 (Table 1). Eight healthy control subjects, matched for age, gender and body weight/fat free mass, were recruited for the respiratory calorimetry studies. For the 31-Phosphorus Magnetic Resonance Spectroscopy ($^{31}$P-MRS) studies, control data from 20 healthy untrained volunteers were used as previously described (8). All subjects were studied after an overnight fast without prior dietary preparation, i.e. on their own diet. The study was approved by the Medical Ethics Committee of our hospital, and written informed consent was obtained from all subjects.

**Methods**

*Respiratory calorimetry.* Open circuit indirect calorimetry was performed at rest under a ventilated hood for 40 minutes. After assessment of height, body weight and fat free mass (four skinfolds) subjects were acclimatized to room conditions (temperature: 21-25 °C) on a bed in the supine position for 30 minutes before beginning the study. Stable values for oxygen consumption ($\text{VO}_2$) were reached within 5 min of recording. Computerized continuous gas analysis and volume measurements ($\text{VO}_2$ and $\text{VCO}_2$) were performed (Oxycon Champion, Jaeger, Breda, The Netherlands), as previously described (9). Gas and volume calibrations were performed before each measurement. Standardization with alcohol burning was performed regularly; the theoretical respiratory exchange ratio (0.67) was closely approached in all standardizations (mean 0.66; coefficient of variation 1.6 percent, n=10). Within subject coefficients of variation for $\text{VO}_2$ measurements (two control subjects, one CID patient; each subject studied three times) ranged from 4.1 to 5.1 percent.

*Maximal exercise testing.* All participants reported to the laboratory to perform an incremental maximal exercise test on an electrically braked cycle ergometer (Lode Instruments, Groningen, The Netherlands). Maximal oxygen uptake ($\text{VO}_2\text{max}$) were assessed as previously decribed (10).

*31$P$-MRS experiments.* Studies were conducted on the superficial mass of the flexor digitorum profundus (FDP) muscle of the right forearm. 31$P$ MRS spectra of the FDP were obtained at 1.5 Tesla on a Philips (Eindhoven, The Netherlands) S15 HP whole-body MRS spectrometer, as decribed in detail elsewhere (11). Briefly, subjects were positioned prone and head-first on
the patient bed with their right forearm extended forward, supported by cushions. Guided by palpation of the ulnar bone directly adjacent to the muscle, the forearm was placed in a support such that the FDP overlied a 25-mm-diameter two-turn surface coil tuned to the $^{31}$P frequency (25.86 MHz) and was attached with Velcro strips. Correct positioning of the FDP over the $^{31}$P surface coil was verified by $^1$H magnetic resonance imaging.

$^{31}$P-MRS spectra of the superficial region of the FDP were obtained with a frequency-modulated adiabatic 90° excitation pulse. 60 free induction decays (FIDs; 1,024 data points with 333 µs dwell time) were collected with a repetition time of 3 seconds. The signals were processed using the LAB ONE (New Methods Research) NMR1 spectroscopy processing software, as described (11). Estimates of the relative peak areas of the various metabolites were obtained by curve fitting of the spectrum to Lorentzian line shapes (P$_i$ and PCr, both singlets; γ-, α-, β-ATP, two doublets and a triplet, respectively). Intracellular pH was determined from the chemical shift of P$_i$ relative to PCr, which was set at 0.00 ppm (12). Any regional variation of pH within the sampled muscle mass was determined by examination of the P$_i$ peak and comparisons of its width with that of the PCr peak, as determined by the fitting routine.

*Calculation of intracellular free ADP concentration and phosphorylation potential*

The average free ADP concentration ([ADP]) in fibers within the sampled muscle mass was calculated from the creatine kinase equilibrium relation according to the equation:

$$[\text{ADP}] = [\text{ATP}] ([\text{TCr}] - [\text{PCr}]) / (1.66 \times 10^9) (10^{-pH}) [\text{PCr}]$$  \[1\]

where $1.66 \times 10^9$ M is the value of the equilibrium constant (13). It was assumed that the concentrations of ATP and total creatine ([TCr]) in skeletal muscle of the patients were unchanged from normal values (8.2 and 42.7 mM for [ATP] and [TCr] respectively, ref. 14). The phosphorylation potential was calculated according to the equation:

$$\text{Phosphorylation potential} = \frac{[\text{ATP}]}{([\text{ADP}] \times [P_i])}$$  \[2\]
Chapter 2

Results

In Table 2 the diagnostic findings are summarized with regard to microscopic and biochemical investigations in freshly biopsied quadriceps (vastus lateralis) muscle of the patients. The muscle biopsy showed ragged red fibers in each of the three patients.

All patients showed severely impaired oxidation of NADH-linked substrates and an enhanced or normal oxidation of FADH$_2$ and cytochrome c-linked substrates. Together these results suggest an isolated defect located at the level of complex I of the respiratory chain. Indeed, the activity of complex I in the muscle homogenate of all patients was severely diminished, while the activities of the complexes III and IV were unimpaired or even increased.

Table 2. Microscopic and biochemical investigation of muscle biopsies in CID patients.

<table>
<thead>
<tr>
<th>CID patient</th>
<th>Controls</th>
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<td></td>
<td>#1</td>
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<tr>
<td>Light microscopy findings</td>
<td>RRF</td>
</tr>
<tr>
<td>Enzyme activity in fresh muscle homogenate [U(+) or mU (-) per g wet weight]:</td>
<td></td>
</tr>
<tr>
<td>- complex I (+)</td>
<td>0.18</td>
</tr>
<tr>
<td>- complex II+III (+)</td>
<td>14.78</td>
</tr>
<tr>
<td>- complex IV (-)</td>
<td>305</td>
</tr>
</tbody>
</table>

Maximal oxygen uptake after substrate stimulation in isolated fresh muscle mitochondria (nanoatoms oxygen min$^{-1}$ per mg protein):

| NADH-linked substrates | | | | | |
| - pyruvate+malate+ADP | 19 | 28 | 9 | 92 ± 5 | 22 |
| - glutamate+malate+ADP | 19 | 30 | 11 | 82 ± 7 | 22 |
| - palmitoylcarnitine+malate+ADP | 21 | 27 | 13 | 68 ± 5 | 22 |

| Other substrates | | | | | |
| - succinate+rotenone+ADP | 166 | 268 | 115 | 104 ± 8 | 22 |
| - ascorbate+TMPD+ADP | 700 | 606 | 329 | 344 ± 13 | 22 |

Enzyme assays and polarographic studies have been performed according to Scholte et al (24). RRF: ragged red fibers, L: lipidosis. TMPD=N,N,N',N'-tetramethyl-$p$-phenylenediamine.
The results of the calorimetric and $^{31}$P-MRS studies as well as the maximal exercise tests are shown in Table 3. Oxygen consumption at rest ($\text{VO}_2\text{rest}$) in each of the three patients was higher than the upper 95% confidence limit of $\text{VO}_2\text{rest}$ in controls, both when expressed per kg of body weight (mean$\pm$SD: 128 ± 14 %, range: 112-137% of values in healthy controls) and per kg of fat free mass (mean$\pm$SD: 127 ± 17%, range: 108-140% of values in healthy controls). There were no differences in respiratory exchange ratio between patients and controls.

Table 3. Results of respiratory calorimetry and $^{31}$P MRS studies in CID patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>CID patient</th>
<th>Controls</th>
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<tbody>
<tr>
<td></td>
<td>#1  #2  #3  mean±SD</td>
<td>mean (95% CI)   n</td>
</tr>
<tr>
<td><strong>Respiratory calorimetry:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{VO}_2$ (ml/min/kg body weight)</td>
<td>3.81 4.61 4.67 4.36±0.48</td>
<td>3.40 (3.31 to 3.49) 8</td>
</tr>
<tr>
<td>$\text{VO}_2$ (ml/min/kg ffm)</td>
<td>4.83 6.25 6.05 5.71±0.77</td>
<td>4.48 (4.36 to 4.60) 8</td>
</tr>
<tr>
<td>$\text{VCO}_2$ (ml/min/kg body weight)</td>
<td>3.11 3.71 4.08 3.63±0.49</td>
<td>2.92 (2.80 to 3.04) 8</td>
</tr>
<tr>
<td>$\text{VCO}_2$ (ml/min/kg ffm)</td>
<td>3.94 5.03 5.29 4.75±0.72</td>
<td>3.85 (3.69 to 4.01) 8</td>
</tr>
<tr>
<td>Respiratory Exchange Ratio</td>
<td>0.81 0.81 0.87 0.83±0.03</td>
<td>0.86 (0.81 to 0.91) 8</td>
</tr>
<tr>
<td><strong>$^{31}$P-MRS:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[\text{PCr}]/[\text{Pi}]$ measured</td>
<td>6.1 5.3 5.3 5.6±0.5</td>
<td>11.1 (10.7 to 11.5) 20</td>
</tr>
<tr>
<td>[ADP] (µmol/L)</td>
<td>24 27 27 26±2</td>
<td>10 (9 to 11) 20</td>
</tr>
<tr>
<td>Phosphorylation Potential (mmol/L$^{-1}$)</td>
<td>57 44 44 48±8</td>
<td>276 (274 to 278) 20</td>
</tr>
<tr>
<td><strong>Maximal exercise testing:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{VO}_2\text{max}$ (ml/min/kg bodyweight)</td>
<td>13.6 12.5 10.5 12.2±1.6</td>
<td>42.9 (41.2 to 44.6) 8</td>
</tr>
</tbody>
</table>

Resting [ADP] and Phosphorylation Potential were calculated from $^{31}$P Magnetic Resonance Spectroscopy ($^{31}$P-MRS) measurements of $[\text{PCr}]/[\text{Pi}]$, recorded from resting forearm muscle (60 free induction decays during four
minutes) as previously described (11), assuming ATP and total creatine concentrations of 8.2 and 42.7 mM respectively (14).

The measured PCR/Pi ratio in resting FDP muscle was below the lower 95% confidence limit of controls in all three patients measured. Their calculated resting ADP concentrations in forearm muscle were markedly higher than the upper 95% confidence limit of controls (mean±SD: 26 ± 2 µM, healthy controls 9 ± 2 µM). Calculated phosphorylation potentials were markedly lower in patients than in controls.

Maximal oxygen uptake (VO$_2$max) was 3.6-, 2.7-, and 2.2-fold increased, when compared with resting VO$_2$ in patient #1, 2, and 3, respectively. Because VO$_2$ increased nearly 13-fold from rest to maximal exercise in controls, VO$_2$max in patients was on average only 28% of VO$_2$max in controls.

Discussion
The present study elaborates on previous incidental observations of increased resting metabolic rates in patients with OXPHOS defects (3,4,5,6). An increased resting metabolic rate seems paradoxical in view of the defective complex I. Three patients with an isolated CID were selected to study this phenomenon in more detail. They were selected on the basis of a relatively mild clinical picture, namely exercise intolerance as their major clinical symptom. We postulated that in these patients the abnormalities in energy metabolism could be studied without interference of more severe consequences of the defect, as seen for instance in patients presenting in the newborn period with overwhelming lactic acidemia.

In our patients we observed that the deficiency of complex I at rest was associated with a significantly elevated resting oxygen consumption rate, a decreased resting phosphorylation potential, and an increased lactate-to-pyruvate ratio in blood. Oxygen consumption during maximal exercise was severely limited. These in vivo findings were accompanied by an increase in the in vitro activity, assayed under optimal conditions, of the distal part of the respiratory chain (CoQ-O$_2$), as well as by an increased amount of mitochondrial protein in their muscle biopsies. These findings suggest that at rest mitochondrial substrate oxidation is stimulated rather than impaired in these three CID patients.
Consequences of the loss of one OXPHOS coupling site. The synthesis of ATP is coupled to respiratory chain activity via the protonmotive force that is generated by proton translocation across the mitochondrial inner membrane at three sites: complex I, III, and IV. In fully coupled mitochondria oxidation of NADH-linked substrates is coupled to proton translocation at all three sites, which results in three ATP synthesized for each oxygen atom reduced (theoretical P/O ratio = 3). Oxidation of FADH$_2$-linked substrates is coupled to only two sites (complex III and IV, theoretical P/O ratio = 2). The P/O ratios that can be measured in ATP synthesizing cells will be lower than these theoretical values following from stochiometry, and have been predicted from non-equilibrium thermodynamics (15). Cairns et al. have actually measured P/O ratios in isolated mitochondria. For the three tissues measured (i.e. brain, liver, and heart) they found average P/O ratios with NADH-linked substrates of 2.32±0.25, whereas with FADH$_2$-linked substrates it was 1.76±0.11 (16). The loss of one OXPHOS coupling site in CID results in a P/O ratio of ~ 1.75 for the oxidation of both NADH- and FADH$_2$-linked substrates (17) and a loss of efficiency of ATP synthesis.

Compensation for the loss of one OXPHOS coupling site. Central to the description of muscle bioenergetics is the phosphorylation potential. In steady-state the phosphorylation potential in the cytosol is the result of the balance between ATP hydrolysis, for maintenance of cellular integrity, and ATP synthesis by mitochondrial OXPHOS. ATP hydrolysis is insensitive towards the cellular phosphorylation potential in resting muscle (18).

By contrast, the rate of ATP synthesis and thereby cellular respiration strongly depends on the phosphorylation potential in resting muscle: it increases sharply with decreasing phosphorylation potential (18). A decrease in the efficiency of ATP synthesis at equal rates of ATP hydrolysis, as in our CID patients, will result in a decrease in the steady-state phosphorylation potential, as was indeed observed. Consequently cellular respiration will increase until a new steady-state is reached, in which the rate of ATP synthesis again matches the rate of ATP hydrolysis. This new steady-state should be reached at a lower phosphorylation potential in order to maintain the increased cellular respiration rate. For our patients it can be calculated that the rate of electron transport (and thus oxygen consumption) should increase to 2.26/1.75 x 100% ~ 130% of controls in order to compensate for the loss of efficiency of ATP synthesis. The observed mean increased resting oxygen consumption rates in these three patients (128±14% of controls, Table 3) compared favourable to this theoretical
value. In addition, the finding of increased activities of the complexes III and IV in muscle of
two of our patients and others (19,20) fits well to the concept of increased electron transport
through the respiratory chain distal of CoQ. An increased rate of electron transport driven by a
decreased phosphorylation potential implies that the rate of electron transport through the
respiratory chain of these patients should be unrestricted at rest. This reasoning tacitly assumes
sufficient residual capacity of complex I to accommodate the increased resting metabolic
demand in these patients. We found that these patients can increase their oxygen consumption
rate (and thus electron transport) almost threefold on average during exercise (Table 3),
suggesting sufficient residual capacity must be present.

An interesting question remains to be elucidated with regard to the increased rate of electron
transport through the respiratory chain in the resting state: where do electrons enter the
respiratory chain in these patients? We would like to emphasize that a defect in complex I does
not necessarily imply a block in the flow of electrons through the complex, but could also
cause impairment of the associated proton translocation across the mitochondrial inner
membrane at this site. Majander et al. have shown that in CID patients respiration (electron
transport) may or may not be impaired depending on the type of mutation involved (21). In the
resting state mitochondrial oxidation of NADH does not seem to be impaired to a significant
extent in our three patients. The increased lactate-to-pyruvate ratios in blood of our patients
indicate that mitochondrial oxidation of NADH however is not completely unaffected.

The question arises why these patients have increased lactate-to-pyruvate ratios at rest,
indicative of an increased cytosolic redox potential (NADH/NAD+), when the rate of electron
flow through the respiratory chain is increased and enough residual capacity seems to be
present. We speculate that this apparent contradiction can be explained by an inverse
relationship between the (increased) redox potential and the (decreased) phosphorylation
potential. Cellular redox state and phosphorylation potential are inversely coupled at the level
of glycolysis by the simultaneous action of glyceraldehyde-3-phosphate dehydrogenase and
phosphoglycerate kinase, and at the level of transport of cytosolic reducing equivalents into the
mitochondria by the energy-dependent malate-aspartate shuttle (22,23).

We conclude that our finding of stimulated rather than impaired substrate oxidation in these
CID patients at rest, points to an increased electron transport rate that may compensate for the
decreased efficiency of oxidative phosphorylation (phosphorylation potential) in these patients.
We speculate that this can result in an increased cytosolic redox potential, as reflected by an increased lactate-to-pyruvate ratio in blood.

Acknowledgements

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References
