High-Throughput Assay to Measure Oxygen Consumption in Digitonin-Permeabilized Cells of Patients with Mitochondrial Disorders

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BACKGROUND: Muscle biopsy analysis is regarded as the gold standard in diagnostic workups of patients with suspected mitochondrial disorders. Analysis of cultured fibroblasts can provide important additional diagnostic information. The measurement of individual OXPHOS complexes does not always provide sufficient information about the functional state of the complete mitochondrial energy-generating system. Thus, we optimized a high-throughput fluorescence-based methodology for oxygen consumption analysis in patient-derived cells.

METHODS: We analyzed mitochondrial respiration in digitonin-permeabilized cells in the presence of a substrate mix containing pyruvate and malate, using a phosphorescent probe, 96-well plates, and a fluorescence plate reader.

RESULTS: In control fibroblasts, we observed clear stimulation by ADP of the pyruvate/malate-driven respiration. Known inhibitors of the OXPHOS system and the Krebs cycle significantly reduced respiration. In patient fibroblasts with different OXPHOS deficiencies, ADP-stimulated respiratory activity was decreased in comparison to control cells. In several patients with reduced ATP production rate in muscle tissue but with normal OXPHOS enzyme activities, the fibroblasts displayed reduced respiratory activity. Finally, we observed a clear difference between control and complex I-deficient transmitochondrial cybrid cells.

CONCLUSIONS: These results confirm the validity of the assay as a high-throughput screening method for mitochondrial function in digitonin-permeabilized cells. The assay allows primary and secondary mitochondrial abnormalities in muscle to be differentiated, which is of great importance with respect to counseling, and also will facilitate the search for new genetic defects that lead to mitochondrial disease.

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Mitochondria play a vital role in cellular energy metabolism. The enzymes of the mitochondrial energy-generating system (MEGS)2 are localized in the mitochondrial matrix space [pyruvate dehydrogenase complex (PDHc) and tricarboxylic acid (TCA) or Krebs cycle] and in the inner mitochondrial membrane [oxidative phosphorylation (OXPHOS) complex] (1). Enzyme analysis of individual OXPHOS complexes in skeletal muscle biopsy remains the mainstay of the diagnostic process for patients suspected of mitochondrial cytopathy (2). In addition, measurement of the MEGS capacity is a powerful tool for the assessment of mitochondrial function. The MEGS capacity can be examined in detail by measurement of 14CO2 production rates from oxidation of [1-14C]pyruvate and carbonyl-14C-labeled TCA cycle intermediates in combination with measurement of ATP production in intact mitochondria from a muscle biopsy (1), or by examining mitochondrial respiration via oxygen consumption measurements using polarography. These methods have been demonstrated to be very valuable in the examination of fresh muscle tissue. For fibroblasts, several methods have been described to measure ATP production or oxygen consumption (3, 4). Analysis of fibroblasts is useful for several reasons (5), including confirmation of the defects observed in muscle, to exclude secondary mitochondrial dysfunction, e.g., due to poor feeding or disuse (6), and serving as a model

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1 Nonstandard abbreviations: MEGS, mitochondrial energy-generating system; PDHc, pyruvate dehydrogenase complex; TCA, tricarboxylic acid; OXPHOS, oxidative phosphorylation; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; SETH, sucrose, EDTA, Tris-HCl, and heparin; ROCR, relative oxygen consumption rate; CS, citrate synthase; RFU, relative fluorescence units; ANT, adenine nucleotide transporter; CCCP, carbonyl cyanide 3-chlorophenyl hydrazone; mtDNA, mitochondrial DNA; ROS, reactive oxygen species.
system for follow-up and functional testing. One of the most informative ways of assessing mitochondrial function is by analysis of mitochondrial oxygen consumption by polarography using the Clark-type oxygen electrode (7). With this method, oxygen consumption can be measured in isolated mitochondria and in intact and detergent-permeabilized cells (8). Although this technique has proven very useful, a major limitation can be the relatively low sample throughput. An assay setup with a higher throughput has recently become available, but requires specialized equipment (9). Quenched-fluorescence oxygen sensing is an approach that may overcome these limitations. This measurement methodology allows the assessment of mitochondrial function using conventional instrumentation, thereby combining the high degree of information provided by oxygen consumption analysis with the simplicity, throughput, and scaling-up capabilities of standard microtiter plate assays (10). This approach (10) has previously been used to investigate mitochondrial toxicity of drugs in isolated mitochondria (7) and in cultured adherent cells (11).

The aim of our study was to adapt a protocol allowing quenched-fluorescence oxygen sensing to be used in the diagnostic evaluation of suspected mitochondrial disorders. We demonstrate that this assay provides a fast and nonlaborious high-content screening method for mitochondrial function in different digitonin-permeabilized cell types.

Materials and Methods

This study was carried out in the Netherlands in accordance with the applicable rules concerning research ethics committee review (Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen) and informed consent.

Materials

We purchased pyruvate and oligomycin from Sigma, malate from Fluka, and ADP from Roche. All other chemicals were of the highest purity commercially available. Phosphorescent oxygen-sensitive probe, MitoXpress™ (A65N-1), was from Luxcel Biosciences. Black-body clear-bottom 96-well plates (Black Isolate TC) and black backing tape were from PerkinElmer.

Cell cultures

After receiving informed consent from patients and their caretakers, we obtained skin biopsy specimens for diagnostic purposes and cultured fibroblasts in E199 medium (Gibco; Invitrogen). We cultured transmitchondrial cybrids in high-glucose (4.5 g/L) Dulbecco’s modified Eagle’s medium (DMEM). Both media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively). Before harvesting, fibroblasts were incubated for 48 h in galactose medium, consisting of DMEM without glucose, without pyruvate, with L-glutamine 2 mmol/L (Invitrogen) supplemented with 10% dialyzed FBS, 1 mmol/L uridine, 1% penicillin/streptomycin, and 5.5 mmol/L D-galactose. Cells were harvested and washed twice in ice-cold 1% FBS in PBS, and we resuspended the cell pellet at 30% (wt/vol) (4–6 × 10^5 cells/μL) in ice-cold 1% FBS in PBS.

Isolation of skeletal muscle mitochondria

We obtained human skeletal muscle from healthy young adults after receiving informed consent. Muscle mitochondria were isolated as described (12). Briefly, we placed the muscle specimens in ice-cold 0.25 mol/L sucrose, 2 mmol/L EDTA, 10 mmol/L Tris–HCl, 5 × 10^4 U/L heparin (SETH) medium. After chopping, homogenization was performed by hand with a Teflon-glass Potter-Elvehjem–type homogenizer. The resulting homogenate was centrifuged for 10 min at 600 g. Mitochondria were isolated from the resulting supernatant by centrifugation for 10 min at 14 000 g. The mitochondrial pellet was finally taken up in SETH-medium.

Fluorescence-based assay of mitochondrial respiration

We dissolved MitoXpress (A65N-1) oxygen probe, supplied as dry reagent in a vial, in PBS to a concentration of 1 μmol/L. Probe fluorescence is quenched by molecular oxygen via a nonchemical (collisional) mechanism, resulting in an increase in probe signal with decreasing concentration of dissolved oxygen due to mitochondrial respiration (10). We performed measurements immediately after harvesting in digitonin-permeabilized nonfractionated cells with the addition of substrates; we also tested isolated fresh human skeletal muscle mitochondria. The incubations were pyruvate + malate with or without ADP. The incubation volume in each well was 50 μL, containing 5 μL cell suspension (2–3 × 10^5 cells), 100 nmol/L fluorescent probe, and a substrate mix consisting of 30 mmol/L potassium phosphate, 75 mmol/L potassium chloride, 8 mmol/L Tris, 1.6 mmol/L EDTA, 5 mmol/L MgCl₂, 0.2 mmol/L p’p’-diphenylphosphatase (myo-adenylate kinase inhibitor), 32.6 μmol/L digitonin for the permeabilization of the cell membranes, 1 mmol/L pyruvate, 1 mmol/L malate, and, where indicated, 2.0 mmol/L ADP, pH 7.4 (1). To regenerate ADP by creatine kinase in the cell preparations, we added 20 mmol/L creatine to all ADP-containing incubations. We added compound solutions to the wells to give the indicated final concentrations. Finally, we added 100 μL mineral oil to each well to seal the sam-
amples from ambient oxygen, which would otherwise de-
stroy oxygen gradients and compromise assay perfor-
mance (10) and placed the plate in a time-resolved
fluorescence plate reader (Viktor 2; PerkinElmer)
equilibrated to a temperature of 37 °C and monitored
every 5 min over 55 min. Instrument settings were 340/
642 nm excitation/emission filters, delay time of 30 μs,
measurement window of 100 μs, and active tempera-
ture control of the microplate compartment at 37 °C.
After completion of fluorescence measurements, time
profiles of fluorescence intensity in each well were an-
alyzed using Viktor (PerkinElmer) and Excel (Mi-
crosoft) software. All measurements were performed in
duplicate and the results averaged. We calculated rela-
tive oxygen consumption rate (ROCR), reflecting re-
spiratory activity, from the maximal linearly increasing
fluorescence intensity slope (Fig. 1) to compare respi-
ration rates of different samples. The results were nor-
malized for citrate synthase (CS) activity (13).

STATISTICAL METHODS
The different fibroblast groups were tested for good-
ness of fit to the normal distribution using the Shapiro–
Wilk test. The Kruskal–Wallis test was applied to study
differences in respiration rates between the investigat-
ged groups. Differences were considered statistically
significant if the $P$ value was lower than 0.05. When
overall significance was found, we used the Mann–
Whitney test with Bonferroni correction ($P$ value
$<0.0125$) as a posthoc test to compare each patient
group to the control.

Results

To evaluate the maximal mitochondrial oxygen con-
sumption capacity of the samples, we did measure-
ments in nonfractionated digitonin-permeabilized
cells under optimal substrate concentrations and in the
presence of an excess of ADP. Most experiments were
performed in fibroblasts to be able to exclude second-
ary mitochondriopathies. Therefore, different media
were tested (data not shown). We used galactose-based
medium, which drives the cell toward the OXPHOS
system for ATP production, thereby increasing the dif-
fences between control and patient fibroblasts
(14, 15), for subsequent experiments in fibroblasts.
Supplemental Table 1 (which accompanies the online
version of this article at http://www.clinchem.org/
content/vol56/issue3) gives an overview of the cell lines
used for oxygen consumption measurements in
fibroblasts.

PYRUVATE-MALATE–DRIVEN OXYGEN CONSUMPTION RATE
In control fibroblasts, pyruvate-malate–driven respi-
ration yielded substantially higher respiratory activity
upon ADP stimulation (Fig. 1A), indicating proper
functional coupling of the mitochondria. This was also
clearly seen in isolated mitochondria obtained from
control fresh muscle biopsies. Muscle showed both a
higher respiratory activity rate and a higher coupling
efficiency than fibroblasts (Fig. 1B). During measure-
ment, the initial reduction in raw signal reflects plate
temperature equilibration (11). This is followed by a
linear phase of oxygen consumption that can be used to
analyze mitochondrial respiration. The linear phase of
oxygen consumption ends owing to oxygen exhaustion
(10), based on results of pyruvate oxidation rate mea-
surements following the methods outlined in Janssen
et al. (1). In cultured control fibroblasts, the pyruvate

![Fig. 1. ADP stimulation of pyruvate-malate–driven respiration in control fibroblasts (A) and isolated mi-
tochondria obtained from a control fresh muscle biopsy (B).](image-url)
Oxygen consumption assay in mitochondrial patient cells

The oxygen consumption rate is between 0.1 and 0.15 nmol/min for 5 μL cells. At this rate, 40 min of pyruvate oxidation produces 16–24 nmol of NADH and reduces 8–12 nmol O₂. In control muscle mitochondria, the pyruvate oxidation rate is 0.55–0.65 nmol/min for 5 μL mitochondrial suspension, producing 28–32 nmol NADH and reducing 14–16 nmol O₂ in 12 min. The addition of a layer of mineral oil to each sample limited back-diffusion of oxygen (10). The presence of low-rate back-diffusion through the mineral oil seal and polystyrene plate (oxygen diffuses through polystyrene at considerable rates) determined the lower limit of assay sensitivity, i.e., mitochondrial oxygen consumption below this rate cannot be analyzed (10). This is in line with previous observations (7, 11). The signal stabilizes when equilibrium is reached between the rates of oxygen consumption and back-diffusion, so successful analysis in the microplate assay requires an oxygen consumption rate sufficient to produce a measurable signal change (10). Because of the back-diffusion, the rates of change of dissolved oxygen measured using this assay are lower than the actual mitochondrial oxygen consumption rate. Therefore, they were expressed as ROCR in relative fluorescence units (RFU) per unit CS. The correlation between initial rates of increase of probe signal and the corresponding mitochondrial protein concentration has been demonstrated to be close to linear (7), which made it possible to compare respiration rates of different samples by analyzing the ROCR of the linearly increasing respiratory activity slope (Fig. 1). Control fibroblasts typically exhibit an ROCR >3 RFU/U CS [mean (SD) 5.0 (2.0)]. We saw that the oxygen consumption rate can decrease when reaching a higher cell culture passage number (Fig. 2 and online Supplemental Table 1).

Effect of inhibitors

To study the sensitivity of the assay, several classical inhibitors of the mitochondrial energy-generating system were added to control fibroblasts: rotenone (for complex I), antimycin A (complex III), azide (complex IV), oligomycin (complex V), atractylside [adenine nucleotide transporter (ANT)], α-hydroxy cyanocinnamate (pyruvate carrier), and arsenite (PDHc and α-ketoglutarate dehydrogenase). Fig. 3A shows that all inhibitors significantly reduced oxygen consumption. Similar results were obtained with isolated mitochondria from a control fresh muscle biopsy (Fig. 3B). This indicates that oxygen consumption measured in this assay depends on a fully functional MEGS system. Moreover, it should be feasible to pick up deficiencies in any of its components.

Diagnostics: Analysis of oxygen consumption in fibroblasts from patients with mitochondrialopathies

For diagnostic purposes, it is important that deficiencies in the MEGS system can be diagnosed or confirmed in fibroblasts. Therefore, we measured patient fibroblasts with various known OXPHOS defects (Fig. 4). Patient cell lines with high passage number and low respiratory activity were not included in this study to prevent possible bias by aging (see Fig. 2). There was an overall difference in respiration rates between the groups (Kruskal–Wallis test, P = 0.002). All complex I– and complex IV– deficient fibroblasts showed an ADP-stimulated respiratory activity rate below the lowest control value. There was a significant difference between the control group and the complex I– (P = 0.001) and complex IV– (P = 0.008) deficient fibroblasts, and no significant difference when comparing the controls with the complex V– deficient fibroblasts (P = 0.427) (Fig. 4). A characteristic feature of the complex V– deficient cells was the clear stimulation of oxygen consumption by addition of an uncoupler [2 μmol/L carbonyl cyanide 3-chlorophenyl hydrazone (CCCP)] (Fig. 5A). This phenomenon was not seen in control cells (Fig. 5B) or cells with other OXPHOS enzyme deficiencies (data not shown). These data indicate that the fluorescence-based oxygen sensing probe assay is a sensitive tool to measure mitochondrial function in fibroblasts.

For patients with non–OXPHOS-related decreases in MEGS capacity in fresh muscle biopsies, the oxygen consumption rate would be an important tool to confirm the deficiency, since traditional OXPHOS enzyme assays are not discriminative. We included sev-
eral fibroblast cell lines from this patient category. The level of oxygen consumption upon ADP stimulation ranged from control respiratory activity rates to predominantly, low to very low rates (Fig. 4). As a group, they were not statistically significant ($P = 0.089$), but nevertheless several of the non–OXPHOS-related MEGS deficiencies could be confirmed in cultured fibroblasts, suggesting that these are most likely caused by 1 or more primary defects.

**CYBRID CELLS**

Because transmitochondrial cybrid cells are often used to verify the pathogenicity of mitochondrial DNA (mtDNA) mutations, we included both control and complex I–deficient (ND1 mutation) cybrid cells. The clear difference in respiratory activity between control and complex I–deficient cybrids (Fig. 6; ROCR after ADP stimulation, 10.1 and 1.1 RFU/U CS, respectively) shows that the assay can also be used to rapidly screen cybrid clones for mitochondrial dysfunction due to mtDNA mutations.

**Discussion**

Despite the availability of many tools to diagnose mitochondrial disease (16, 17), current methods are still insufficient for the evaluation of a large subgroup of patients suspected to have a mitochondriopathy. Here, we show that quenched-fluorescence oxygen sensing provides a fast and efficient additional diagnostic tool that can be used to examine the mitochondrial energy-generating system in cultured patient-derived cells.

The assay showed a clear difference between control and patient cell lines: control cells showed a sub-
Oxygen consumption assay in mitochondrial patient cells

A substantial increase in oxygen consumption rate upon ADP stimulation, in contrast to the patient cell lines. This was illustrated in particular by the cybrid cells. Complex I– and complex IV– deficient fibroblasts showed a significantly decreased oxygen consumption rate. Complex V– deficient fibroblasts could be recognized because of a substantial increase after uncoupling. Addition of an uncoupler leads to a decrease in the mitochondrial membrane potential, which subsequently gives rise to a maximal stimulation of the respiratory rate (18). Uncoupling makes the oxygen consumption rate independent of complex V, and this explains the initial increase in complex V– deficient cells. It has also been shown that CCCP stimulates apoptosis via loss in mitochondrial transmembrane potential and reactive oxygen species (ROS) production (19). Cell death, combined with back-diffusion of oxygen, could explain the subsequent decrease of the respiratory activity slope that was sometimes observed. Inhibitors of the entire pathway from pyruvate import to ATP export, and several steps in between (the MEGS), reduced oxygen consumption in fibroblasts and isolated muscle mitochondria, confirming that the integrity of the entire OXPHOS machinery can be tested successfully in both permeabilized cells and isolated mitochondria. A peculiar finding was a slight loss of inhibition of oligomycin in the fibroblasts after 35 min; this was not seen in isolated muscle mitochondria. Further research is needed to understand this finding.

Some cautionary notes must be taken into account when measuring the oxygen consumption rate in fibroblasts. We saw that the rate can decrease when reaching a higher cell culture passage number (Fig. 2 and online Supplemental Table 1). To get reliable results, it is very important to measure cells at an early passage stage and to measure samples in at least 2 independent experiments. Usually, the passage number increases by 1–2 between measurements. In case of poor reproducibility of multiple measurements with increasing passage number, a possible negative effect of the passage stage should be considered. Results should then be distrusted and cells from an earlier passage should be tested, if available.

Fig. 5. Complex V– deficient fibroblasts showed clearly higher rates after the addition of an uncoupler (10 mmol/L CCCP) (A), a phenomenon not seen in control cells (B).

Normalized time profile of fluorescence intensity; 8836, complex V– deficient fibroblasts; 8088, control fibroblasts; ○, + ADP; □, − ADP; ▼, + ADP + CCCP; △, − ADP + CCCP.

Fig. 6. Analysis of oxygen consumption in cybrid cells showing a clear difference between control and complex I– deficient transmitochondrial cybrid cells.

Normalized time profile of fluorescence intensity; 873, control cybrids, cy ND1, complex I– deficient cybrids due to the mutation m.3460G>A in the MT-ND1 gene (known LHON mutation); ●, cy 73 + ADP; ○, cy 73 − ADP; ▼, cy ND1 + ADP; △, cy ND1 − ADP.
The assay can also be used to support the search for new defects in mitochondrial energy metabolism. In a group of patients with decreased ATP production in fresh muscle, a subgroup could be identified with clearly reduced oxygen consumption rate, despite normal OXPHOS enzymes and PDHc activities. Such data indicate that another still-unidentified defect in the mitochondrial energy metabolism may be responsible for the reduced oxygen consumption rates. This will encourage further studies to uncover new genetic defects causing mitochondrial disease. Another subgroup of patients showed normal or near-normal oxygen consumption rates in fibroblasts that may be a reflection of a tissue-specific mitochondriopathy, which is not uncommon, e.g., mtDNA mutations with uneven tissue distribution or POLG [polymerase (DNA directed), γ, formerly POLG1] gene mutations. A normal oxygen consumption rate in fibroblasts can also be expected when the MEGS dysfunction in muscle tissue is secondary (poor feeding, disuse) (6). Differentiating between primary and secondary mitochondrial abnormalities is of great importance with respect to counseling. Thus, this assay provides a valuable tool in the diagnostics of mitochondrial disorders. Advantages of the assay compared to substrate oxidation measurements include its simplicity, the requirement of less cell material, and the elimination of the need of an isotope laboratory. When comparing oxygen-sensitive fluorescence-based methods to classical polarography, it has been shown that, due to the low rate of back-diffusion, the rates of change of dissolved oxygen measured using the microplate approach are lower than the consumption rates measured using a sealed polarographic system (7).

In conclusion, quenched-fluorescence oxygen sensing provides a simple, high-throughput, high-content screening method for mitochondrial function in different cell types. It requires a relatively low sample volume and allows for simultaneous testing of multiple assay conditions. In addition, this approach is also suitable for the analysis of cell lines with relatively low mitochondrial activity (e.g., fibroblasts). Patients with a primary mitochondriopathy that does not affect the complexes of the respiratory chain can be identified. In combination with functional studies and other diagnostic assays (for instance, homozygosity mapping), the availability of this approach will facilitate the search for hitherto-unknown genetic defects leading to mitochondrial disease.

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