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Assay of mitochondrial respiratory chain complex I in human lymphocytes and cultured skin fibroblasts

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Abstract

Respiratory chain complex I (NADH:ubiquinone oxidoreductase) deficiency is one of the most frequent causes of mitochondrial disease in humans. The activity of this complex can be confidently measured in most tissue samples, but not in cultured skin fibroblasts or circulating lymphocytes. Highly contaminating non-mitochondrial NADH–quinone oxidoreductase activity in fibroblasts and the limited access of substrates to complex I in lymphocytes hinder its measurement in permeabilized cells. Complex I assay in these cells requires the isolation of mitochondria, which in turn necessitates large quantities of cells and is not feasible when studying circulating lymphocytes. Here we report a simple method to measure complex I activity in a minute amount of either cell type. The procedure strongly reduces contaminating NADH:quinone oxidoreductase activity and permits measuring high rates of rotenone-sensitive complex I activity thanks to effective cell permeabilization.

Keywords: Human; Mitochondria; Respiratory chain; Complex I assay; Lymphocytes; Fibroblasts

NADH:ubiquinone oxidoreductase or complex I is the largest mitochondrial respiratory chain complex, with at least 36 subunits in human [1]. It is therefore not surprising that defects affecting complex I activity represent a significant portion of respiratory chain diseases, up to a third of our experience [2]. Complex I deficiencies are detected by both polarographic and spectrophotometric assays in skeletal muscle mitochondria, or by spectrophotometric assays in homogenates prepared from skeletal muscle, heart, liver, and kidney micro-biopsies [3]. The identification of a series of mutations in both nuclear and mitochondrial genes encoding complex I subunit further strengthens the usefulness of a biochemical characterization of complex I deficiency [4–6]. However, the measurement of complex I is not easily performed in cultured skin fibroblasts (or cultured trophoblasts) or circulating lymphocytes, requiring either isolation of mitochondria from a large amount of cells [7] or indirect estimation by polarographic means [3]. In skin fibroblasts, this is due mainly to the presence of a highly contaminating NADH:ubiquinone oxidoreductase activity—which may represent up to 90% of the measured NADH oxidation rate in cultured skin fibroblasts. Difficulties to assay complex I may also arise from a limited permeability to complex I substrates, particularly in lymphocytes. The situation in cultured skin fibroblasts (and cultured trophoblasts) is particularly worrying because these cells (fibroblasts and trophoblasts for the probant and the fetus, respectively) have to be used to propose a biochemical prenatal diagnosis of this condition [8], as the molecular basis of complex I deficiency is seldom identified [9].

After several years of unsuccessful attempts, we have now devised a simple method allowing for measurement of complex I activity using a quite small number of cultured skin fibroblasts or circulating lymphocytes.

Materials and methods

Patient. Cultured skin fibroblasts were obtained from healthy controls and one patient born to non-consanguineous healthy parents.

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He presented with Leigh-like syndrome. Its clinical presentation has been previously reported [6].

Cells. Cultured skin fibroblasts were grown under standard conditions and circulating lymphocytes were isolated on a Ficoll cushion as previously described [3]. A small aliquot of the cell pellet (fewer than 1 million cells; 0.5–0.7 mg protein) was deep-frozen in 50 μl phosphate buffered saline solution and subsequently thawed using 1 ml of ice-cold solution (medium A) consisting of 0.25 M sucrose, 20 mM Tris (pH 7.2), 40 mM KCl, 2 mM EGTA, and 1 mg/ml bovine serum albumin, 0.01% digitonin (w/v), and 10% Percoll (v/v). After 10 min incubation at ice temperature, cells were centrifuged (5 min × 5000 rpm), the supernatant was discarded, and the pellet was washed (5 min × 8000 rpm) with 1 ml of medium A devoid of digitonin and Percoll. The pellet was next re-suspended in 30 μl of medium A and used for enzyme assays. NADH:ubiquinone reductase and additional respiratory chain enzyme activities were spectrophotometrically measured as previously described [3]. Polarographic assays were carried out as described [3]. Protein was measured according to Bradford [10].

Results and discussion

A hypotonic shock has been previously found to both selectively destabilize the contaminating NADH:ubiquinone oxidoreductase and to break open the mitochondria to NADH in skeletal muscle and in most human tissue homogenates allowing measurement of complex I activity [11]. However, we found hypotonic shock insufficient to decrease the contaminating activity in freeze-thaw permeabilized fibroblasts or to allow easy access of the NADH and/or decylubiquinone to mitochondrial CI in freeze-thaw permeabilized circulating lymphocytes (Table 1). We therefore attempted to permeabilize plasma membranes to both contaminating soluble enzymes and to solutes by using a small concentration of digitonin (0.01% w/v). This digitonin concentration is known to selectively destabilize the cholesterol from the plasma membrane without affecting mitochondrial membranes [12], and has been used routinely for the past few years in our polarographic assay medium to study the substrate oxidation by both fibroblast and lymphocyte mitochondria [13]. In addition, we added 10% Percoll to the permeabilization medium since poly-vinyl-pyrollidone-covered silica has been shown to readily trap soluble proteins [14], such as those presumably responsible for non-mitochondrial NADH:ubiquinone oxidoreductase activity. After treatment for 10 min with both digitonin and Percoll, cells were spun down, washed, and assayed for rotenone-sensitive complex I activity. As shown in Table 1, this procedure allowed us to measure in both cell types a significant rate of rotenone-sensitive activity while significantly lowering the rotenone-resistant activity measured in fibroblasts. Noticeably, all respiratory chain enzyme activities bound to mitochondrial membranes were also found to be measurable on such preparations. In particular, the activity of the oligomycin-sensitive ATP hydrolase (mitochondrial respiratory chain complex V) could be estimated in the same cuvette subsequently to complex I activity assay, after addition of the components required to perform the standard coupled ATPase assay using pyruvate kinase and lactate dehydrogenase (Fig. 1). Finally, the procedure was successful using both unfrozen and frozen cells (not shown).

Malate oxidation and CI activity were next studied in cultured skin fibroblasts derived from a patient presenting Leigh-like syndrome resulting from a G13513A mutation in the ND5 gene of the mitochondrial DNA. Malate oxidation, measured in the presence of glutamate, was found to be significantly decreased (Fig. 2, traces a

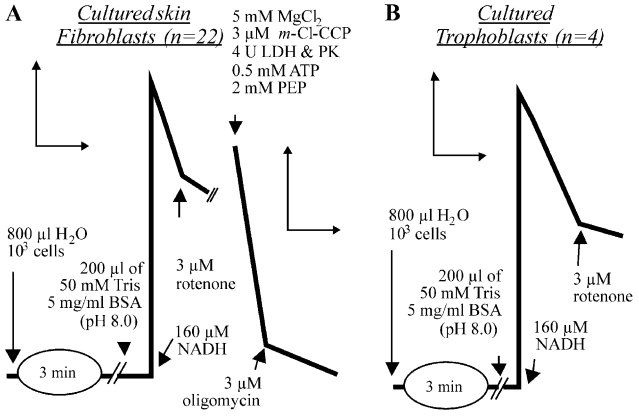

Fig. 1. Spectrophotometric assay of complex I (NADH quinone reductase) activity in cultured skin fibroblasts and trophoblasts. Assay as described under Materials and methods. Numbers along the traces are mean values ± 1 SD.

Table 1
The NADH:ubiquinone reductase activities in cultured skin fibroblasts and circulating lymphocytes

	NADH:ubiquinone oxidoreductase activity (nmol/min/mg protein)		
	Total	Rotenone-resistant	Rotenone-sensible (complex I activity)
Fibroblasts			
Frozen/thawn ($n = 3$)	260 ± 30	250 ± 26	12 ± 4
Digitonin- and Percoll-treated ($n = 22$)	32.9 ± 6.1	4.0 ± 0.4	28.2 ± 6.2
Lymphocytes			
Frozen/thawn ($n = 3$)	4.0 ± 0.2	n.d.	n.d.
Digitonin- and Percoll-treated ($n = 6$)	22.6 ± 5.0	5.0 ± 0.6	17.6 ± 5.0

Enzyme activity was assayed either on frozen cell samples or after permeabilizing and washing cells in a medium containing both digitonin and Percoll as described under Materials and methods.

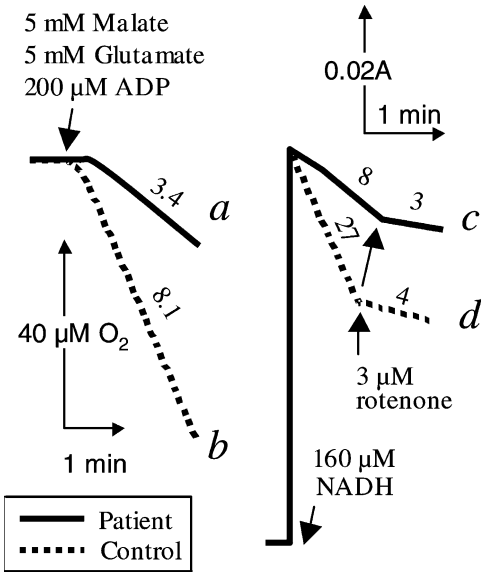

Fig. 2. Polarographic and spectrophotometric analyses of complex I dependent activities in patient (a, c) and control (b, d) cultured skin fibroblasts. Assay as described under Materials and methods. Numbers along the traces are either nmol oxygen consumed/min/mg protein (traces a, b) or nmol NADH oxidized/min/mg protein (traces c, d).

and b). Accordingly, the assay of complex I activity revealed a similar loss of activity (Fig. 2, traces c and d).

This study thus reports on an improved procedure to measure mitochondrial respiratory chain complex I activity using a small number of cells. It applies to both frozen and fresh cell samples. Among respiratory chain diseases, those originating from complex I deficiency are relatively frequent and requests for prenatal diagnosis have been hindered by both the difficulty encountered in measuring complex I activity and the scarcity of cases with an identified molecular diagnosis. We therefore hope to provide a simple and reproducible assay for complex I in human cells allowing on one hand a better detection of complex I deficiency in circulating lymphocytes and, on the other hand, easier detection in cultured cells (fibroblasts and trophoblasts) in order to open new possibilities for prenatal diagnosis of this condition.

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