Diagnosis of Complex I Deficiency in Patients with Lactic Acidemia Using Skin Fibroblast Cultures

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The requirement for a rapid and easy method of preparing mitochondrial fractions from cultured skin fibroblasts led us to compare the results obtained from such a preparation with the more traditional methods of cellular fractionation. Values for NADH-cytochrome c reductase (rotenone sensitive) were compared for a series of three controls and nine patients with complex I (NADH-coenzyme Q reductase deficiency). Values obtained for deficient cell lines varied from 19 to 64% of the control values for the long mitochondrial preparation method and from 34 to 70% of control for the rapid preparation. Mean values were statistically significantly different from the lowest control cell line (P < 0.01) in all cases. The specific activity on the basis of activity per milligram of mitochondrial protein and of activity per unit of citrate synthase activity was lower in the rapid preparation of mitochondria by some 41%, indicating a lesser degree of mitochondrial purification. However, the overall result showed that this type of rapid preparation, which uses four 9-cm petri dishes of cultured cells, can be used to diagnose mitochondrial complex I deficiency. This method will find general use in the measurement of either mitochondrial enzymes of low specific activity or mitochondrial enzymes whose measurement is made difficult by contaminating nonmitochondrial enzymes.

MATERIALS AND METHODS

NADH-CoQ reductase (complex I deficiency) is the most difficult of the mitochondrial disorders to diagnose with confidence. While the activity of this enzyme in isolated muscle mitochondria is largely rotenone sensitive and thus easy to measure, any damage to the mitochondria during isolation can result in the destruction of the complex and misdiagnosis. In other tissues, such as liver or fibroblasts, the presence of large amounts of diaphorase leads to difficulties with the NADH-CoQ reductase assay because the diaphorase catalyzes a rapid nonrotenone-sensitive reaction of this type which exceeds the activity of the respiratory chain enzyme activity, sometimes by a factor of 100:1 (1,2). In addition to this problem, the distinction between secondary deficiencies of complex I caused by mitochondrial DNA mutations as a result of impairment of mitochondrial protein synthesis and primary deficiencies caused by nuclear or mitochondrial encoded defects in the protein subunits of the complex is not an easy one to make in muscle. Cultured skin fibroblasts rarely express these tRNA mtDNA mutations to any extent because of reduced heteroplasmy, so they can be used as a vehicle for the diagnosis of isolated primary complex I deficiency (3–6).

Here we examine the possibilities of using a rapid fibroblast mitochondrial preparation method for the estimation of the extent of complex I deficiency by measurement of rotenone-sensitive NADH-cytochrome c reductase and show that this may be used to make the diagnosis, albeit with lower overall specific activities for the complex.

All purchased chemicals were the best grade available. All required protein concentrations were assayed by Lowry et al. (7).

Skin fibroblast cultures. Human skin fibroblasts were grown from explants of forearm skin biopsy tissue (taken with informed consent). Culture medium was Eagle’s alpha minimum essential medium supplemented with 10% fetal calf se-
rum and extra glucose to bring the final medium glucose to 10.5 mm.

Lactate to pyruvate ratios. Ratios were determined as described previously (8) by determining levels of lactate and pyruvate in the incubation medium after a 1-h period of glucose metabolism in Krebs' phosphate buffer containing 1 mm glucose.

Respiratory chain activities and citrate synthase. Whole-cell cytochrome oxidase (complex IV) was measured as described by Glerum et al. (3) and succinate cytochrome c reductase (complexes II and III) by following the reduction of cytochrome c at 550 nm by the method of Fischer et al. (2). Mitochondria were isolated from cultured skin fibroblasts and activities of the rotenone-sensitive NADH:cytochrome c reductase (complexes I and III) were measured by the method of Moreadith et al. (4). Citrate synthase was measured by the method of Shepherd and Garland (9).

Patients. Patients were initially selected on the basis of elevated lactate to pyruvate ratios in skin fibroblasts (10) and normal whole-cell activities of succinate cytochrome c reductase and cytochrome oxidase, thus having the tentative diagnosis of complex I deficiency. All patients exhibited elevated blood lactic acid levels. Three patients had Leigh's disease, two had fatal infantile cardiomyopathy with cataracts, and two had hepatopathy and tubulopathy. Two further patients with cataracts and developmental delay were included on the basis of deficient complex I activity in muscle, despite having normal lactate to pyruvate ratios in cultured skin fibroblasts.

Preparation of skin fibroblast mitochondria. The two methods used to make mitochondrial fractions from skin fibroblasts are set out in diagram form in Fig. 1. In the long preparation 15 petri dishes (9 cm diameter) of cells were processed by initially washing the cells with 0.25 M sucrose, 20 mM Tris-HCl, and 1 mM EDTA buffer, pH 7.4. The cells were then scraped using a rubber policeman from the dishes and homogenized with 20 strokes of a glass on teflon homogenizer. The homogenate was centrifuged at 12,000 rpm for 5 min in order to get the cell debris down, and the supernatant was centrifuged at 10,000 rpm for 5 min. The mitochondrial pellet was then resuspended in 0.5 ml sucrose buffer. The samples were sonicated for 5 s just before use.

RESULTS

Patients were initially selected on the basis of elevated lactate to pyruvate ratios and normal whole-cell activities of succinate cytochrome c reductase and cytochrome oxidase, thus having the tentative diagnosis of complex I deficiency. These initial results are published elsewhere by our group, together with details of clinical presentation (5). Briefly, the patient cohort consisted of two patients with fatal cardiomyopathy and cataracts, two patients with hepatopathy and tubulopathy, two patients with mild developmental delay, and three patients with Leigh's disease. Only the patients with mild developmental delay and one patient with hepatopathy and tubulopathy survive to this date.

The objective of this study was to evaluate the rapid mitochondrial method outlined above as a vehicle for the diagnosis of complex I deficiency. The activity of the rotenone-sensitive NADH-cyto-

![Preparation of Fibroblast Mitochondria](image)

**FIG. 1.** The two different methods to make mitochondrial fractions from skin fibroblasts. This diagram shows the essential steps in the rapid preparation method versus the long preparation method for cultured skin fibroblast mitochondria.
We showed that the preparation of a mitochondrial fraction from cultured skin fibroblasts using a rapid fractionation procedure would give values for NADH–cytochrome c reductase (rotenone sensitive) that would allow for the diagnosis of partial complex I deficiency. Determination of the activity in a rapid preparation of mitochondria with each determination repeated three times provides a value for NADH–cytochrome c reductase (rotenone sensitive) that in most cases of complex I deficiency provides a reliable measurement of the extent of the deficit. The advantages of this procedure are numerous: the preparation takes as little as 30 min from start to finish compared with 2 h for the traditional preparation; the time and cost of preparation that ipso facto goes into subculturing cells as starting material for the procedure are reduced significantly by a factor of 4/15; the procedure produces a mitochondrial fraction which gives similar ratios of NADH–cytochrome c reductase to citrate synthase, showing that the amount of mitochondrial breakage is similar in the two preparations; and this procedure is adaptable to prenatal diagnosis for complex I deficiency.

Before setting up this procedure there was no reliable measurement of the activity of cytochrome c reductase (complexes I and III) in the mitochondrial respiratory chain was found to vary from 19 to 64% of the mean of the control values by the long mitochondrial preparation method (Fig. 2). Values for the rapid mitochondrial preparation method varied from 34 to 70% of control values (Fig. 3). While the range of percentage deficit was not greatly changed, the specific activity of the complex I and III measurement was reduced by 50% in the short preparation method, for both the patients and the controls. The activity of citrate synthase to the rotenone-sensitive NADH–cytochrome c reductase was measured for the short and long preparation methods simultaneously in three patients and two controls in order to determine if either preparation method resulted in a loss of soluble mitochondrial matrix enzymes by breakage compared to the membrane-bound respiratory chain complex. When this was done it was found that the specific activity of both of these enzymes was lower in the short preparation method, but that the ratio of the activities of complexes I and III to citrate synthase was relatively constant (Fig. 4). The rapid preparation method actually gave higher NADH–cytochrome c reductase activities relative to citrate synthase, suggesting that there is actually some loss of matrix enzyme in the rapid preparation method.
RAPID PREPARATION OF FIBROBLAST MITOCHONDRIA

chondrial enzymes or as a measurement of mitochondrial enzymes with low activity in skin fibroblasts. In this regard we found that this method was very adaptable for the measurement of succinyl CoA-3-ketoacid transferase, an enzyme which is difficult to measure in whole-cell extracts of skin fibroblasts.

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REFERENCES


