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Defective Intramitochondrial NADH Oxidation in Skin Fibroblasts from an Infant with Fatal Neonatal Lacticacidemia

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SUMMARY

A small-for-gestational-age female infant born at term developed severe lactic acidosis and died on day 13 of life. Two previous sibs had also died of overwhelming lactic acidosis in the neonatal period. The lactate-to-pyruvate and 3-hydroxybutyrate-to-acetoacetate ratios were elevated at 136 and 42 to one, respectively. The activities of the pyruvate dehydrogenase complex and pyruvate carboxylase in cultured skin fibroblasts were normal but a defect in respiration was indicated by the low rates of conversion of 1-[^{14}C]pyruvate, glutamate, and lactate to $^{14}CO_2$ in these cells. Skin fibroblast cultures also displayed an elevated lactate-to-pyruvate ratio (72:1) when incubated with glucose as substrate compared to control cell cultures (20:1). When mitochondrial preparations of skin fibroblasts (prepared by digitonin extraction) were tested for their ability to synthesize ATP from a variety of substrates, it was found that those of the patient made adequate amounts of ATP with either succinate or ascorbate/tetramethyl-phenylenediamine as substrate but not with the NAD-linked substrates pyruvate, isocitrate, and palmitoyl carnitine. We propose that this is indicative of a defect in the respiratory chain between NADH and coenzyme Q, for the first time demonstrable in cultured skin fibroblasts.

INTRODUCTION

Inborn errors of metabolism that have been reported as leading to overwhelming lacticacidemia in the neonatal period are deficiencies in the pyruvate dehy-

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drogenase complex and in pyruvate carboxylase activity [1–4]. In such cases, the lacticacidemia results from an inability of the affected individual to utilize pyruvate. In other cases, a disturbance in the redox equilibrium of the cell may lead to lacticacidemia [5] because of excessive intracellular shunting of pyruvate to lactate. Another group of children in this category have been described as having muscle cytochrome oxidase deficiency [6–9], and some patients with chronic lacticacidemia have been described as having muscle respiratory chain defects [10–14]. This latter group of patients is unusual in that the defects described in muscle are not demonstrable in fibroblasts, and, in some cases, the fibroblasts appear to be normal in every respect. One exception to this is the case of Miyabayashi et al. [15] who was a patient having exercise intolerance and a partial deficiency of cytochrome oxidase present in both muscle tissue and in cultured skin fibroblasts.

Here, we describe the use of techniques to investigate cellular redox state and mitochondrial metabolism leading to the identification of a respiratory chain defect in a strain of cultured skin fibroblasts.

CASE REPORT

The patient (K. V.) was a small-for-gestational-age female infant born at term and noticed to be feeding poorly during the first week of life. On day 10, the patient developed tachypnea; by day 12, the patient was exhibiting Kussmaul breathing and apneic episodes. Metabolic acidosis was documented with a plasma bicarbonate of 6 mM, serum lactate was 30 mM (normal 0 to 3–1.2), and serum pyruvate was 0.22 mM (normal 0.03–0.09 mM). In addition, serum 3-hydroxybutyrate was 1.5 mM, while serum acetoacetate was 0.04 mM. Serum alanine was also elevated at 1.69 mM (normal 0.24–0.35 mM). Blood ammonia was in the normal range. On day 13, the child died of overwhelming acidosis. A skin fibroblast culture was established. Two previous sibs had died of overwhelming lactic acidosis in the neonatal period. A preliminary report of this case has been published in abstract form [16].

MATERIALS AND METHODS

Skin fibroblast cultures were grown in α-MEM medium supplemented with 15% fetal calf serum. Pyruvate dehydrogenase was assayed in the native and dichloroacetate-activated states by the method of Sheu et al. [17] as modified by Hyland and Leonard [18], pyruvate carboxylase by the method of Ballard and Hanson [19], and phosphoenolpyruvate carboxykinase by the method of Atkin et al. [20]. Whole cell pyruvate oxidation was measured by the method of Robinson et al. [21].

Assessment of ATP Production in Digitonin-treated Fiboblasts

Skin fibroblast cultures at or near confluence in 9-cm Petri dishes were drained of culture medium by siphon and the medium immediately replaced with 1 ml of a medium containing 0.25 M sucrose, 20 mM MOPS (morpholinopropane sulphonate), pH 7.4, and 0.8 mg/ml digitonin. After 3 min, this was removed by suction and replaced by 1 ml of a solution containing 0.25 M sucrose, 20 mM EDTA (ethylenediamine tetraacetate), and 20 mM MOPS. After 5 min, this medium was replaced with 1 ml of a medium containing 0.25 M sucrose, 25 mM MOPS, pH 7.4, 1 mM EDTA, 5 mM potassium phosphate, and 1 mM ADP (no substrate medium). To assess the potency of various substrates with respect to ATP production, various substrates were added to this basic medium. After various time intervals of 15 min, 30 min, and 1 hr, samples were removed, treated with

0.05 vol of 1.6 M perchloric acid, and centrifuged to remove protein, and the resulting extract assayed by enzyme fluorimetric methods for ATP [22].

Assessment of Lactate/Pyruvate Production in Skin Fibroblast Cultures

Confluent skin fibroblast cultures (~ 1 mg cell protein) were drained of culture fluid and the medium replaced with a Krebs phosphate buffer (5 ml). The fibroblast cultures were then incubated for 1 hr at 37°C to deplete substrate and glycogen reserves when the Krebs phosphate buffer was replaced by 2 ml of Krebs phosphate buffer containing 1 mM glucose. After 1 hr, 0.1 ml 1.6 M perchloric acid was added and the fluid removed from the Petri dish and assayed for lactate and pyruvate [2] after centrifugation.

RESULTS

Assay of enzyme activities in cultured skin fibroblasts revealed that the pyruvate dehydrogenase complex, pyruvate carboxylase, and phosphoenolpyruvate carboxykinase were all in the normal range. However, oxidation of 1-[^{14}C]pyruvate to $^{14}CO_2$ by intact fibroblasts was deficient (table 1) but the defective oxidation was relieved by the addition of methylene blue. Oxidation of 1-[^{14}C]glutamate and 1-[^{14}C]lactate was also deficient.

When cultured skin fibroblasts were incubated in a Krebs phosphate medium with 1 mM glucose, they produced lactate at the rate of about 600 nmol/min per mg cells and pyruvate at the rate of 25 nmol/min per mg cells, giving a lactate-to-pyruvate ratio of 25 to 1 (table 2). In the cells from a patient with a severe deficit (< 5% of normal activity) in the pyruvate dehydrogenase complex (cell strain 1373), the ratio was similar. In our patient (cell strain 1680), the amount of pyruvate produced was much lower than in other cell lines, giving a much

TABLE 1

ENZYME AND OXIDATIVE CAPACITIES IN CULTURED SKIN FIBROBLASTS

	Patient	Controls
	(nmol/min/mg protein)	
Pyruvate dehydrogenase native	0.476 ± 0.066 (3)	0.557 ± 0.155 (3)
Pyruvate dehydrogenase DCA activated	0.558 ± 0.046 (3)	0.710 ± 0.119 (3)
Pyruvate carboxylase	1.28 ± 0.25 (4)	1.08 ± 0.28 (4)
Phosphoenolpyruvate carboxykinase	2.22 ± 0.6 (3)	3.51 ± 1.07 (3)
	(nmol/hr/mg protein)	
Whole-cell 1-[^{14}C]pyruvate oxidation	3.9 ± 0.9 (7)*	18.1 ± 2.7 (7)
plus methylene blue 50 μM	21.4 ± 3.5 (4)	24.2 ± 3.7 (4)
Whole-cell 1-[^{14}C]glutamate oxidation	3.0 ± 0.2 (16)*	(a) 8.8 ± 0.3 (6)
		(b) 9.0 ± 0.5 (6)
		(c) 15.5 ± 0.3 (4)
Whole-cell 1-[^{14}C]lactate oxidation	1.0 ± 0.2 (30)*	(a) 15.1 ± 2.5 (15)
		(b) 14.6 ± 1.1 (14)
		(c) 20.1 ± 1.8 (15)

NOTE: Results are expressed as the mean ± SEM, the no. observations being given in parentheses. For glutamate and lactate oxidation control, values are given for three separate control cell strains (a), (b), and (c).
* Result significantly different from controls, $P = < .005$.

TABLE 2

LACTATE-TO-PYRUVATE RATIOS IN CONFLUENT SKIN FIBROBLAST CULTURES METABOLIZING 1 mM GLUCOSE

Cell strain		Lactate produced	Pyruvate produced	Lactate/Pyruvate ratio
		(nmmol/hr/mg cell protein)		
Control	1685	620 ± 58 (9)	25.0 ± 4.7 (9)	24.8 ± 2.7 (9)
	1206	586 ± 92 (5)	20.7 ± 4.0 (5)	28.3 ± 3.1 (5)
	1228	569 ± 53 (3)	33.6 ± 3.2 (3)	16.9 ± 3.7 (3)
PDH deficient	1373	710 ± 58 (8)	33.4 ± 4.7 (6)	21.2 ± 3.8 (6)
Propositus	1680	753 ± 137 (8)	10.5 ± 1.9 (8)**	71.7 ± 5.3 (8)*
Control	1685 + rotenone (1 μM)	827 ± 51 (5)*	6.2 ± 1.7 (5)***	133.3 ± 23 (5)*

NOTE: Experiments were carried out as described in the MATERIALS AND METHODS section. Results are expressed as the mean ± SEM, the no. determinations are given in parentheses. Significance is designated: *$P = < .01$; **$P = < .005$; ***$P = < .001$, all with respect to control cell strain 1685.

TABLE 3

ATP PRODUCTION IN DIGITONIN-TREATED FIBROBLASTS WITH SUBSTRATES FOR MITOCHONDRIAL RESPIRATION

Additions	Controls	Cell strain 1373	1680 patient
	(nmol ATP/per hr/mg protein)		
No substrate (zero time) ...	28.3 ± 4.1 (6)	27.2 ± 3.5 (3)	21.4 ± 4.4 (4)
No substrate (1 hr)	49.3 ± 5.8 (8)	45.2 ± 6.7 (4)	29.2 ± 2.3 (4)
2 mM ascorbate/TMPD 0.1 mM (1 hr)	107.3 ± 8.2 (5)	84.5 ± 6.2 (4)	110.1 ± 7.2 (4)
5 mM succinate/rotenone 1 μM (1 hr)	174.8 ± 15.3 (7)	180 ± 29.7 (7)	154 ± 21 (5)
5 mM pyruvate/L-malate 0.3 mM (1 hr)	160.6 ± 18.7 (10)	46.5 ± 5.1 (6)*	33.5 ± 4.2 (6)*
1 mM isocitrate/0.1 ml L-malate (1 hr)	150.0 ± 20.4 (7)	145.5 ± 13.1 (4)	30.1 ± 3.1 (6)*

NOTE: Experiments were carried out as described in the MATERIALS AND METHODS section, with substrates added to the incubation medium as indicated. Results are expressed as the mean ± SEM, the no. determinations are given in parentheses.

* Result statistically different from controls, $P = < .001$.

higher lactate-to-pyruvate ratio of 72:1. Adding 1 μM Rotenone to control cells elevated the lactate-to-pyruvate ratio to a higher level than that seen in cell strain 1680.

When isolated cells are treated with digitonin at a concentration of 0.8 mg/ml, they become permeable to cytosolic metabolites and proteins that then escape into the surrounding medium [23]. In cultured skin fibroblasts, we have used this technique to investigate mitochondrial metabolism. Cell cultures that have been digitonin-treated and washed can be microscopically observed to have retained their nuclei, cytoskeleton, and mitochondria but the cell membranes are indistinct. When we supplied such a permeabilized cell culture with ADP and inorganic phosphate and no substrate, a small amount of ATP was synthesized in a 1-hr period. When a substrate suitable for mitochondrial metabolism was added, the permeabilized cells made ATP in a linear fashion for 2 hrs (results not shown). Succinate, pyruvate/malate, and isocitrate/malate were almost equal in their ability to support ATP synthesis (table 3). Ascorbate/tetramethylphenylenediamine (TMPD) would also support ATP synthesis at substantial rates, but since its oxidation only supports phosphorylation at one site, it cannot equal the rates of ATP synthesis with the NADH or flavoprotein-linked substrates. When this system was tested with cell strain 1373, a pyruvate-dehydrogenase-deficient cell strain [4], values for ATP synthesis were comparable except for the case when pyruvate was the substrate. With this cell strain, ATP synthesis with pyruvate was no better than with no substrate at all. The permeabilized cell culture from our patient showed slightly lower endogenous levels of ATP at zero time, very little ATP synthesis with no added substrate, and normal ATP synthesis with both ascorbate/TMPD and succinate, but no ATP synthesis with either pyruvate or isocitrate. This was also the situation with palmitoyl carnitine as substrate (results not shown). The content

of NAD and NADH in the mitochondria of cell strain 1680 was the same as in control cell lines (6 nmol/mg mitochondria).

DISCUSSION

The clinical history of the patient indicated that we were dealing with a situation in which a disordered redox state was associated with severe lacticacidemia. The lactate/pyruvate ratio in the serum of greater than 100:1 and the concurrent 3-hydroxybutyrate/acetoacetate ratio of 37.5:1 are indicative of very low NAD/NADH ratios in both the cytosolic and mitochondrial intracellular compartments. This situation might be indicative of either hypoxia, a primary problem in the mitochondrial redox system, or a secondary problem in redox states such as has been described associated with pyruvate carboxylase deficiency [3]. That this problem might involve a defect in respiration was demonstrated by the low oxidation rates with 1-[^{14}C]pyruvate, lactate, and glutamate as substrate in the cultured cells, while the enzymes of pyruvate metabolism were found to have normal activity. The oxidative defect in these cells was also found to be quite reversible by methylene blue, a situation described previously for two cell strains from children with abnormal redox states [5]. Methylene blue is an electron acceptor that can accept from both cytoplasmic and mitochondrial NAD and NADP linked redox systems so that the defect could be located in either compartment.

That a disordered redox state was also present in the cultured fibroblasts from this patient was demonstrated by the abnormal lactate-to-pyruvate ratio when the cells metabolized glucose. Interestingly, although the cell strain from the patient produced only slightly more lactate than did a normal cell strain, it manifests the redox problem in a lower pyruvate production rate. Adding rotenone, a respiratory inhibitor, produced a similar mode of behavior in control cells, that is, lactate production increased with a threefold change in redox state. This is not surprising since skin fibroblasts derive most of their energy from glycolysis by preference [24] and inhibition of respiration has little effect on glucose utilization. The site of the defect in cell strain 1680 is shown clearly by the experiments with ATP synthesis in digitonin permeabilized cells. This preparation can be treated very much like a mitochondrial preparation in terms of activity. It shows that although ATP synthesis can occur at sites II (between CoQ and cytochrome c) and III (between cyt c and cytochrome oxidase) of the respiratory chain assembly, it cannot occur with any substrate that is NAD-linked and oxidized through site I (between NADH and coenzyme Q) (fig. 1). This tentatively places the lesion between NADH and coenzyme Q. It could be brought about by a lack of NAD in the mitochondrial compartment, but two circumstances suggest that this is unlikely. First, the NAD and NADH content of the isolated mitochondria from cell strain 1680 is no different from other cell strains. Second, the relief of constraint of oxidation by methylene blue of pyruvate oxidation suggests an intact working pyruvate dehydrogenase complex with a normal complement of NAD.

Attempts by us to demonstrate unequivocally a defect in rotenone-sensitive NADH cytochrome c reductase in skin fibroblasts and skin fibroblast mito-

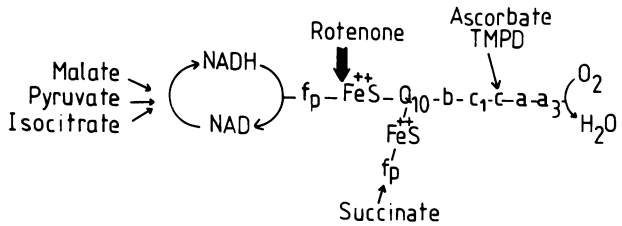

FIG. 1.—The mitochondrial respiratory chain is depicted to show the site of input of reducing equivalents from: (1) pyruvate, malate, and isocitrate, (2) succinate, (3) ascorbate/TMPD. The site of inhibition by rotenone is shown by the *thick arrow*.

chondria have been unsuccessful. The finding of very high levels of NADH-cytochrome b_5-cytochrome c reductase in both cell extracts and fibroblast mitochondrial preparations makes it impossible to monitor accurately the rotenone-sensitive component. A similar defect in the respiratory chain was reported recently in which identification of the lesion was performed in isolated kidney and liver mitochondria, but not in fibroblasts [14].

Respiratory-chain components are synthesized either in the cytosol and then imported into the mitochondria or they are synthesized in the mitochondria [25]. Such proteins are coded for in the first instance by nuclear DNA or in the second case by mitochondrial DNA. The mitochondrially encoded components that have been identified consist of two subunits of the oligomycin-sensitive ATPase and three subunits of cytochrome oxidase and cytochrome b [25, 26]. So far, no component of complex I has been identified as being encoded by the mitochondrial genome. This segment of the respiratory chain between NADH and coenzyme Q has at least 25 different polypeptide components [27], and it is quite possible that one or more of these polypeptides is encoded by the mitochondrial genome since there are at least five unassigned reading frames in the human mitochondrial genome [28]. If the defect in this patient were to reside in a mitochondrially encoded protein, this would dictate maternal inheritance of the defect. Since both parents are unaffected and there is one normal sibling, this is unlikely to be the case.

Here, we have demonstrated that this type of defect can be detected in fibroblasts and that further approaches of this nature, or perhaps the use of immunoprecipitation techniques, might lead to methods for the prenatal diagnosis of these disorders. If the defect can be identified early enough as a respiratory-chain defect, it would be important in future cases to obtain a transformed lymphocyte culture. This, because of the greater ease of culture and the provision of greater amounts of material with which to work, would allow biochemical investigations in greater depth.

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