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Lactate Oxidation for the Detection of Mitochondrial Dysfunction in Human Skin Fibroblasts

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To screen fibroblasts for defects in lactate/pyruvate oxidation, cells were grown to confluence in 25-cm^2 flasks, rinsed, and incubated in glucose-free media containing 25 μM L-lactate and 0.1 μCi [D,L-1-^{14}C]lactate. Lactate oxidation was measured as the amount of lactate oxidized in nmol of $^{14}CO_2$ generated /mg protein/min. Fibroblasts from patients with mitochondrial or peroxisomal disorders had decreased lactate oxidation compared to the control (CON): CON, 1.9 ± 0.13 nmol/mg/min; neonatal adrenoleukodystrophy (NALD), 0.45 ± 0.01 ($P < 0.001$); rhizomelic chondrodysplasia punctata (RCDP), 0.13 ± 0.002 ($P < 0.001$); mitochondrial defect of unknown etiology (MIT), 0.77 ± 0.003 ($P < 0.001$); pyruvate dehydrogenase (PDH) deficiency, 0.98 ± 0.02 ($P < 0.001$). This method is useful for screening fibroblasts for defects in lactate oxidation in patients with mitochondrial or peroxisomal disorders. Confirmation of the site of the defect may then be investigated with specific assays, e.g., PDH, in cellular homogenates: CON, 0.93 ± 0.02 nmol/mg/min; NALD, 0.55 ± 0.02; RCDP, 0.44 ± 0.02; MIT, 0.53 ± 0.03; PDH deficiency, 0.19 ± 0.02. © 1993 Academic Press, Inc.

Type B_3 lactic acidosis occurs in patients with inherited disorders of enzymatic defects in gluconeogenesis, pyruvate oxidation, citric acid cycle, and electron transport (1,2). Some of the enzymes involved, i.e., electron transport system (complexes I, III, IV, and V), include enzyme complexes whose individual subunits may be encoded by mitochondrial DNA, nuclear DNA, or both (3). The two most common defects responsible for type B_3 lactic acidosis include pyruvate oxidation defects secondary to decreased pyruvate dehydrogenase (PDH)[1] activity (1,2) and electron transport defects. PDH (EC 1.2.4.1) is a regulatory enzyme located in the inner mitochondrial membrane and represents the rate-limiting step for the flow of pyruvate from the cytoplasmic glycolytic pathway to the citric acid cycle within the mitochondria. PDH is a multienzyme complex which catalyzes the oxidative decarboxylation of pyruvate to acetyl CoA and CO_2 (4). The activity of the enzyme complex is regulated by phosphorylation (inactive) and dephosphorylation (active) at three sites on the alpha subunit of the E1 component (5).

Screening assays using cultured skin fibroblasts have been developed to detect mitochondrial dysfunction including electron transport defects, citric acid cycle defects, and PDH deficiency. Defects in electron transport have been evaluated using the ratio of lactate/pyruvate generated from glucose (6–9), [2-^{14}C]pyruvate oxidation (10–12), or monitoring the mitochondrial respiration-dependent electrochemical potential with a cationic amphiphilic dye (13). Flux through the citric acid cycle has been monitored using the ratios of $^{14}CO_2$ produced from two isotope pairs, [2-^{14}C]pyruvate:[3-^{14}C]pyruvate and [1-^{14}C]acetate:[2-^{14}C]acetate (14). The conversion of [1-^{14}C]pyruvate to $^{14}CO_2$ by fibroblasts with or without the addition of the PDH kinase inhibitor, dichloroacetic acid, is the most common screening method used for possible PDH defects (10–12,15,16). However, [1-^{14}C]pyruvate is relatively unstable and is subject to nonenzymatic decarboxylation which will increase the background level of $^{14}CO_2$ (10,17,18) decreasing assay reliability, especially in fibroblasts with low levels of PDH activity.

In order to rapidly evaluate mitochondrial function in intact fibroblasts from patients with peroxisomal disorders and lactic acidosis, we have used [1-^{14}C]lactate as

[1] Abbreviations used: PDH, pyruvate dehydrogenase; NALD, neonatal adrenoleukodystrophy; RCDP, rhizomelic chondrodysplasia punctata; MIT, mitochondrial defect of unknown etiology; CCCP, carbonyl cyanide chlorophenylhydrozone; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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a substrate. The [1-^{14}C]lactate is converted by the fibroblasts to [1-^{14}C]pyruvate by lactate dehydrogenase and finally to $^{14}CO_2$ by PDH. Lactate oxidation and PDH activity are both decreased in fibroblasts from patients with four different causes of type B_3 lactic acidosis: two peroxisomal disorders, neonatal adrenoleukodystrophy (NALD) and rhizomelic chondrodysplasia punctata (RCDP), and two mitochondrial disorders, mitochondrial defect of unknown etiology (MIT) and PDH deficiency. Results of these studies have been partially presented elsewhere (19–21).

MATERIALS AND METHODS

Materials. Sodium [D,L-1-^{14}C]lactate (55 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Scintillation fluid, Ready Safe, was from Beckman. L-lactic acid, lactate dehydrogenase, carbonyl cyanide chlorophenylhydrozone (CCCP), sodium fluoride, dichloroacetic acid, rotenone, dimethylsulfoxide, D-glucose, bovine insulin, and other reagents were from Sigma (St. Louis, MO).

Tissue culture. Human skin fibroblasts were obtained by skin biopsy from the two pediatric patients with peroxisomal disorders and one patient with a mitochondrial disorder after obtaining informed consent from the parents and following guidelines established by the William Beaumont Hospital Human Investigations Committee. Human skin fibroblasts obtained from remnants of human foreskin removed from healthy infants undergoing routine circumcision were used as controls.

Biopsy specimens were subcultured in minimum essential medium (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (FBS) (Biocell, Carson, CA), 50 U/ml penicillin G potassium, 50 μg/ml streptomycin sulfate (GIBCO, Grand Island, NY), and 100 μU/ml insulin (Sigma, St Louis, MO). Fibroblasts were grown as monolayer cultures and maintained in a 37°C humidified incubator containing a mixture of 95% air and 5% CO_2. Media was replaced twice a week and cells passed during log growth phase. Cultures were routinely screened for Mycoplasma contamination by Mycotrim TC (Hanna Biologicals Inc., Alameda, CA). Fibroblasts were transferred for only 15 passages. The PDH deficient fibroblast cell line GM03093 was purchased from National Institute of General Medical Sciences cell repository and cultured under the same conditions.

The above media was prepared without glucose and insulin for the lactate oxidation assay. The sodium bicarbonate was replaced with 50 mM potassium phosphate with final pH 7.4. FBS was present at the time of the assay unless otherwise indicated.

Lactate oxidation assay. Metabolism of [D,L-1-^{14}C]-lactate was measured in fibroblasts grown to confluence (except when cell number is designated) in 25-cm^2 tissue flasks containing stock media (described above). Cells were rinsed twice in phosphate-buffered saline (PBS) and then preincubated for 1 h in glucose-free media. The assay was initiated by the addition of 1 ml glucose-free media containing 25 μM L-lactate (Sigma, St Louis, MO), and 0.1 μCi/ml [D,L-1-^{14}C]lactate (American Radiochemical Co.) and the flasks slowly shaken at 37°C from 30 min to 2 h 40 min depending on the assay. Filter paper moistened with 0.2 ml of 100 g/liter NaOH, used to collect released $^{14}CO_2$, was suspended directly above the monolayer in each flask with an air-tight stopper. Each filter was transferred into scintillation vials and the amount of $^{14}CO_2$ collected was determined. The cells were then harvested for protein determination. Activity was expressed as nmol $^{14}CO_2$ released per milligram cellular protein per minute. Background activity, determined using flasks without cells and containing 25 μM L-lactate and 0.1 μCi/ml [D,L-1-^{14}C]lactate in 1 ml glucose-free medium was subtracted from both the control and the patient results. Cell number was determined with a Coulter (Model ZM) counter and total protein determined by the bicinchoninic assay (22).

The final concentration of the mitochondrial complex I respiratory chain enzyme inhibitor, rotenone, prepared in 0.2% (v/v) dimethyl sulfoxide, was 25 μM. The final concentration of CCCP, a mitochondrial electron chain uncoupler, was 20 μM. Mitochondrial metabolism was also investigated after addition of 5 mM glucose or insulin (1–1000 μU/ml) which were added to the glucose-free medium just prior to the assay.

PDH assay. The PDH assay is modified from that of Schofield *et al.* (23). Approximately 50 million cells were washed and harvested 3–4 days after reaching confluence by gentle scraping into homogenization buffer containing 25 mM NaF, 4 mM dichloroacetic acid, 1 mM dithiothreitol, 1 mM EGTA, and 250 mM sucrose in 40 mM phosphate buffer (pH 7.4). Filter paper moistened with 0.2 ml of 100 g/liter NaOH was suspended in a vial with an air-tight stopper directly over the assay mixture. The assay mixture containing 50 mM potassium phosphate buffer (pH 7.4), 2.5 mM $MgCl_2$, 1.5 mM β-nicotinamide adenine dinucleotide (β-NAD), 500 μM $CaCl_2$, 80 μM thiamine pyrophosphate, 100 mU/ml phosphotransacetylase, 0.2 mM coenzyme A, 1 mM dithiothreitol, and the fibroblast suspension (20–100 μl) containing 0.05–0.25 mg protein was preincubated for 10 min at 37°C. The reaction was started by adding 0.1 μCi/ml [D,L-1-^{14}C]lactate, 1.1 mM L-lactate, and 10 IU L-lactate dehydrogenase to a total volume of 0.5 ml. The reaction was stopped after 30 min at 37°C by injecting 6 N H_2SO_4 into the assay mixture. Incubation continued for 30 min at 37°C, and then the vial was opened and the filter paper transferred to scintillation fluid and counted. Experiments were performed in triplicate.

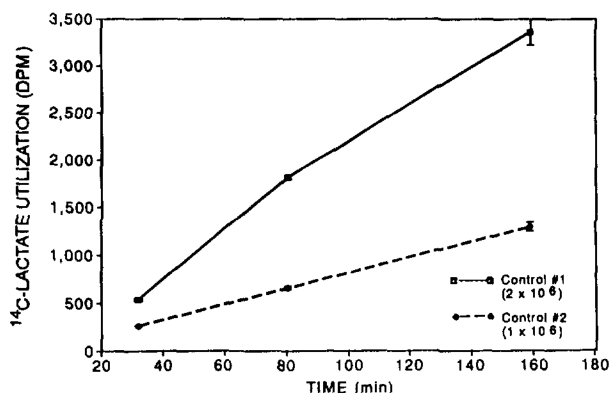

FIG. 1. Effects of increasing cell number and assay time on lactate oxidation in human skin fibroblasts from pediatric control subjects. Cell number was determined, then the appropriate number were transferred to 25-cm^2 flasks and allowed 16 h to attach. To measure the rate of lactate oxidation, cells were washed and incubated in glucose-free minimum essential medium containing 25 μM L-lactate and 0.1 μCi [D,L-1-^{14}C]lactate for 1 h (Methods). Control 1 with 2×10^6 cells per flask was compared to Control 2 with 1×10^6 cells per flask and the results expressed as dpm of substrate transformed into $^{14}CO_2$. Experiments were performed in triplicate and the results represent the mean ± SE.

Blank values were obtained from identical assay tubes without CoASH and β-NAD. PDH activity is expressed as nmol $^{14}CO_2$ released per milligram of homogenate protein per minute. Values were expressed as the mean ± SE. Statistical significance was assessed by the unpaired Student t test and defined as $P < 0.05$.

RESULTS

The amount of $^{14}CO_2$ generated from [D,L-1-^{14}C]-lactate is dependent on cell number and incubation time (Fig. 1). The rate of lactate oxidation is directly related to cell number: control 2, 8 dpm $^{14}CO_2$/min for 1×10^6 cells; control 1, approx. 20 dpm $^{14}CO_2$/min for 2×10^6 cells. Confluent cells represent a total of 2×10^6 to 3×10^6 cells. All subsequent assays were performed on fibroblasts 4 days after they reached confluence.

The amount of $^{14}CO_2$ generated is also dependent on the composition of the media (Table 1); the lactate utilization by cells was decreased significantly in the presence of 5 mM glucose and increased to 121% ($P < 0.05$) of control by the addition of 100 μU/ml insulin. In Table 1, the addition of rotenone (25 μM) inhibits lactate oxidation to 8.1% ($P < 0.001$) of control and CCCP (20 μM) stimulates lactate oxidation to 155% ($P < 0.001$) of control.

In Table 2, lactate oxidation is significantly decreased compared to the control in the fibroblasts from patients with known mitochondrial dysfunction, MIT to 40% ($P < 0.001$) of control and the PDH deficiency to 51% ($P < 0.001$) of control. The lactate oxidation from patients with peroxisomal disorders was decreased compared to the control NALD to 24% ($P < 0.001$) and RCDP to 6.8% ($P < 0.001$) suggesting mitochondrial dysfunction. The activity of the specific mitochondrial enzyme PDH was decreased compared to the control in fibroblast homogenates from all four disorders: NALD, 59% ($P < 0.001$); RCDP, 47% ($P < 0.001$); MIT, 57% ($P < 0.001$); and PDH deficiency, 20% ($P < 0.001$).

TABLE 1

Effects of Agents Which Alter Mitochondrial Metabolism on Lactate Oxidation

Fibroblasts[a]	Lactate oxidation (nmol/mg/min)		P value
Control	1.9	±0.13	
Insulin (100 μU/ml)	2.3	±0.14	<0.05
CCCP (20 μM)	2.95	±0.07	<0.001
Rotenone (25 μM)	0.15	±0.03	<0.001
Glucose (5 mM)	0.62	±0.03	<0.001

[a] Control human fibroblast (confluent) was incubated in glucose-free medium containing 25 μM L-lactate and 0.1 μCi [D,L-1-^{14}C]lactate for 60 min. Results represent the mean ± SE of experiments performed in triplicate.

DISCUSSION

The lactate/pyruvate oxidation assay measures the release of $^{14}CO_2$ from [1-[D,L-1-^{14}C]lactate added to skin fibroblasts grown to confluence. Figure 1 demonstrates, in the absence of glucose, lactate oxidation in human skin fibroblasts is directly related to the cell number and incubation time. When compared to different cell types,

TABLE 2

Measurement of Lactate Oxidation and Pyruvate Dehydrogenase Activity in Fibroblasts

	Activity (nmol/mg/min)	
Fibroblasts	Lactate oxidation	Pyruvate dehydrogenase
Controls	1.90 ± 0.01	0.93 ± 0.02
Mitochondrial disorders		
PDH deficient cells	0.98 ± 0.02*	0.19 ± 0.02*
MIT patient	0.77 ± 0.003*	0.53 ± 0.03*
Peroxidomal disorders		
NALD patient	0.45 ± 0.01*	0.55 ± 0.02*
RCDP patient	0.13 ± 0.002*	0.44 ± 0.02*

Note. Lactate oxidation in human fibroblasts from control and patients with four different disorders. Fibroblasts with significantly lower lactate utilization include two mitochondrial disorders (PDH deficiency and MIT) and two peroxidomal disorders (NALD and RCDP). Experiments were performed in triplicate and the results represent the mean ± SE.

* $P < 0.01$.

i.e., CHO, BC3H-1, and HL-60, the human skin fibroblasts demonstrate a low rate of lactate oxidation (24). Since glucose has been depleted for an hour prior to the addition of radiolabeled lactate, the main substrate for pyruvate metabolism is lactate and the constant rate of lactate utilization suggests that the metabolic flux through PDH is constant. The assay measures a physiological basal level of PDH activity which can be increased by the addition of CCCP, an electron chain uncoupler, or insulin, known to stimulate PDH activity (11,25) (Table 1). Maximal PDH activity could be estimated by the addition of the PDH kinase inhibitor, dichloroacetic acid (16).

The addition of glucose in Table 1 increases the concentration of pyruvate through glycolysis and decreases the activity of lactate dehydrogenase, shifting the equilibrium of the reaction toward the formation of lactate (26). This shift in equilibrium causes an increase in the conversion of pyruvate to lactate, diluting the labeled lactate available for conversion to pyruvate.

The dramatic decrease in lactate oxidation associated with the addition of rotenone (Table 1), a complex I mitochondrial respiratory chain inhibitor, demonstrates the interrelationship between oxidative phosphorylation and PDH activity. When the rotenone was rinsed from the cells and rotenone-free culture media containing [D,L-1-^{14}C]lactate was added, lactate oxidation increased by 50% during the next hour and had returned to control levels by 24 h (data not shown).

The actual control value for lactate oxidation of 1.90 nmol/mg protein/min determined in newborn fibroblasts is higher than PDH activities of 1.23 nmol/mg protein/min determined by Sheu *et al.* (27) using the untreated newborn control fibroblast homogenates or the homogenate of the NaF-DCA-treated control fibroblasts of 0.93 nmol/mg protein/min (Table 2). Sheu *et al.* (27) suggested that their method may underestimate PDH activity secondary to artifacts introduced during isolation and handling of the cells. For example, glucose oxidation of fibroblasts in culture is inhibited 82% following removal of the cells from the culture flask by scraping (28). Sheu *et al.* (27) estimates that the rate of pyruvate oxidation may be higher than their [1-^{14}C]-pyruvate oxidation measurements in fibroblasts under physiological conditions. Whether these values reflect an accurate measurement of basal PDH activity or whether these values reflect altered PDH activity secondary to glucose depletion or other factors is still under investigation. It has been reported that basal PDH activity is less than 20% of the maximum enzyme activity (11,27).

Similar problems may explain the differences in lactate oxidation and PDH activity measured in fibroblasts from the four patients with type B_3 lactic acidosis (Table 2). The patients reported here with NALD and RCDP have demonstrated increased concentrations of plasma lactic acid (19–21). The fibroblasts from both patients demonstrate significantly decreased lactate oxidation and PDH activity. Patients with NALD and RCDP usually exhibit defects in lipid metabolism including increased intracellular concentrations of very long chain fatty acids, very long chain acyl-CoAs and altered plasmalogen synthesis (29). These metabolic dearrangements may lead to defective architecture of the inner mitochondrial membrane, changing the three-dimensional configuration of the PDH complex and inactivating it (12). The two patients with mitochondrial disorders (MIT and PDH deficiency) are expected to demonstrate decreased lactate oxidation and PDH activity. However, some patients with known electron transport deficiencies, like mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episode, have failed to exhibit altered fibroblast metabolism of lactate or pyruvate (8,9). This finding may be secondary to the heterogeneity of mitochondrial DNA defects expressed in a variety of tissues in the same patient (30). Therefore, fibroblasts may not exhibit the mitochondrial DNA defect observed in other tissues. Likewise, fibroblasts will not detect PDH deficiency in patients where the defect in enzyme activity is limited to the brain (31), but will detect those patients with "cerebral" lactic acidosis where 5/6 patients had decreased PDH activity in skin fibroblasts (32). Understanding these limitations, the lactate oxidation assay is a reliable rapid screening method for evaluating potential mitochondrial dysfunction in patients with mitochondrial or other disorders.

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