Determination of Pyruvate Oxidation Rate and Citric Acid Cycle Activity in Intact Human Leukocytes and Fibroblasts

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We measured pyruvate oxidation in intact leukocytes and fibroblasts by measuring 14CO2 production. The optimal pyruvate concentration appeared to be higher than that usually applied. Activities remained constant during the incubation and were proportional to the amount of tissue protein added. Mean values (± SD) were 2.8 ± 0.9 nmol/h per 10^6 cells and 37 ± 14 nmol/h per mg of protein for leukocytes and fibroblasts, respectively, for [1-14C]pyruvate oxidation; and 2.1 ± 0.8 nmol/h per 10^6 cells and 18 ± 7 nmol/h per mg of protein, respectively, for [2-14C]pyruvate oxidation. We compared oxidation rates of pyruvate and 2-oxoglutarate by intact cells with those of isolated mitochondria. The ratio of 14CO2 production vs. activity of mitochondrial marker enzyme demonstrated that the rate of pyruvate oxidation can adequately be assayed in intact cells, but that the permeability of the cell membrane is rate-limiting in the oxidation of 2-oxoglutarate. No significant oxidation of other intermediates of the citric acid cycle was found, presumably owing to a low rate of transport of these substances across the cell membrane.

Additional Keyphrases: pyruvate oxidation · use of intact cells to measure enzyme activity · transport across the cell membrane · 2-oxoglutarate · pediatric chemistry · lactic acidemia

During the last years there has been increasing interest in determining enzyme activities in cultured fibroblasts and leukocytes. Though enzyme defects can be established in liver, kidney, or muscle biopsy materials, these studies are rather limited. Frequently only fibroblasts are available for enzyme assays. Because leukocytes can easily be obtained, they are also an important source for the study of enzyme activity. We have preferred to work with intact cells, so as to approach physiological conditions insofar as possible. Use of intact cells has the advantage that the intracellular relationships are intact. Moreover, the cytoplasm and the intra-mitochondrial content are diluted on sonication, which necessitates addition of cofactors to the incubation medium. Our procedure is superior to most of the previous methods because deficiencies of cofactors may be established as well.

Our aim in the present investigation was to study the contribution of transport across the cell membrane in the assay of pyruvate oxidation with [1-14C]pyruvate and the citric acid cycle activity with [2-14C]pyruvate and [1-14C]2-oxoglutarate in intact leukocytes and fibroblasts.

Materials and Methods

Leukocytes

One volume of 50 g/liter dextran solution (T500; Pharmacia, Uppsala, Sweden) containing, per liter, 7 g of NaCl and 15 g of disodium ethylenediaminetetraacetate was added to five volumes of blood. Blood samples were obtained after an overnight fast from children (1–10 years), all hospitalized for disorders not interfering with pyruvate metabolism. The total leukocyte count was in the range 5·10^9 to 10·10^9 per liter. The differential leukocyte count was: 60–70% neutrophils, 20–30% lymphocytes, and 2–10% monocytes. After 30 min at 4 °C the supernatant plasma—containing leukocytes, some erythrocytes, and platelets—was centrifuged for 8 min at 300 X g. The supernatant plasma, which contained most of the platelets, was discarded. The pellet was suspended in 1 ml of buffered saline (40 mmol/liter phosphate buffer, pH 7.4, containing 0.11 mol of NaCl per liter). The remaining erythrocytes were removed by applying an hypotonic osmotic shock by exposure to distilled water for 90 s, after which isotonicity was restored (J). After washing with saline, the final pellet was suspended in buffered saline and the cells were counted in a Coulter Counter.

Mitochondria were prepared from a concentrated suspension of leukocytes (30·10^9/liter) in 0.25 mol/liter of sucrose. The cell membrane was disrupted with a French press at 3.5 X 10^6 Pa. The cell debris was removed by centrifugation (10 min at 70 X g) and the mitochondria obtained by centrifugation for 13 min at 8000 X g were washed once with 0.25 mol/liter of sucrose.
Fibroblasts

Skin biopsy material was cut into small fragments and cultured in TC-199 medium (Flow Laboratories, Irvine, Scotland) enriched with, per liter, 200 g of fetal calf serum and 50 g of chicken embryo extract, supplemented with 1 × 10^6 USP units of penicillin and 100 mg of streptomycin. After trypsinization for 5 min at 37 °C (2.5 g of trypsin per liter; Difco, Detroit, Mich.; 1:250 pancreas) the fibroblasts were washed with the phosphate-buffered saline. The cells were counted under the microscope in a special glass counting chamber after dilution of the cell suspension.

For isolation of mitochondria, fibroblasts were suspended in a medium containing, per liter, 0.27 mol of mannitol, 0.1 mmol of disodium ethylenediaminetetraacetate, 0.5 g of bovine serum albumin, and 10 mmol of tris(hydroxymethyl)aminomethane-HCl (pH 7.3). The cells (4-10^9/liter) were disrupted at 4 °C with a Potter–Elvehjem homogenizer and the mitochondria were isolated by differential centrifugation for 7 min at 770 × g and 10 min at 10 000 × g.

Enzyme Assays

Leukocytes (5 × 10^6 to 10^7) were incubated in the phosphate-buffered saline and either pyruvate or 2-oxoglutarate (0.5 mmol/liter), the final volume being 2.1 ml. The specific activity of the used substrates was 1.0 mCi/mol. Incubation was done in a shaking water bath at 37 °C in 20-ml glass scintillation vials, sealed with rubber stoppers, containing two small tubes, one fitting inside the other (2). The reaction was stopped after 60 min by injecting 0.3 ml of 3 mol/liter of perchloric acid and the 14CO2 produced was trapped in Hyamine hydroxide [p-(disobutyl-cresoxyethoxyethyl)dimethylbenzylammonium hydroxide] during further incubation for 30 min at 37 °C. The trapped 14CO2 was measured after adding 10 ml of scintillation fluid (4 g of Omnifluor per liter of toluene) in a liquid scintillation counter. For the determination of the blank values the cells were omitted.

Fibroblasts (5 × 10^5 to 10^6) were incubated under identical conditions as described for leukocytes except for the substrate concentrations of pyruvate and 2-oxoglutarate (0.2 mmol/liter). The final volume was 1.5 ml. Protein was assayed according to Lowry et al. (3).

Mitochondria

Pyruvate oxidation by mitochondria isolated from fibroblasts and leukocytes was measured at 37 °C in an incubation mixture containing, per liter, 50 mmol of potassium phosphate buffer (pH 7.4), 0.5 mmol of disodium ethylenediaminetetraacetate, 2 mmol of ADP, 75 mmol of KCl, 10 mmol of MgCl2, 1 mmol of the substrate (0.5 mCi/mol), and 0.2–0.4 mg of mitochondrial protein. The final volume was 0.5 ml. Incubation time was 60 min.

Leukocytes and fibroblasts yielded about 4 and 40 mg of mitochondrial protein per 10^6 cells, respectively. The assay procedure was as described for intact cells.

Cytchrome c oxidase (EC 1.9.3.1) activity and glutamate dehydrogenase (EC 1.4.1.2) activity were measured in cells and mitochondria, both after freezing and thawing three times. These assays were performed according to Cooperstein and Lazarow (4) and Schmidt (5), respectively.

The respiratory control index of mitochondria of fibroblasts was measured in simplified Dow (6) medium containing 1 mmol of pyruvate and 1 mmol of malate per liter. ADP was added in a final concentration of 0.2 mmol/liter.

Radiolabeled Materials

[1-14C]Pyruvate, [2-14C]pyruvate, and [1-14C]2-oxoglutarate were obtained from the Radiochemical Centre, Amersham, and stored at −20 °C in small aliquots as the dry sodium salts, under nitrogen.

Results

Isolation of Leukocytes and Fibroblasts

The differential leukocyte count remained unchanged after isolation in comparison with that in the peripheral blood. Recovery of leukocytes in the final preparation averaged 50–60%; 6% of the original amount of platelets was found in the final leukocyte suspension, which contained on the average equal amounts of erythrocytes and leukocytes.

After trypsinization, 2–5% of the fibroblasts were non-viable, as judged by trypan blue uptake.

Isolation of Mitochondria

Leukocytes are relatively resistant to the usual homogenization procedures (7). The best disruption of cells was obtained by a more vigorous method: use of a French press. The respiratory control index of the mitochondria of leukocytes could not be established because of the low oxidative capacity.

Fibroblasts could be well homogenized with a Potter–Elvehjem homogenizer in isotonic medium. The respiratory control index of the isolated mitochondria was 4.0 with pyruvate/malate as substrates.

Enzyme Assays

14CO2 production from [1-14C]pyruvate was lower without added cells than that in the presence of boiled cells. Nearly identical blank values were obtained either in the absence of cells or after inhibition of pyruvate oxidation with arsenite (4.4 mmol/liter). The presence of boiled cells appeared to give an incorrect blank value by a higher nonenzymatic decarboxylation of [1-14C]-pyruvate (8).

The results (Figure 1) indicate that the reaction rate of the oxidation of [1-14C]pyruvate and [2-14C]pyruvate by fibroblasts remained constant during 60 min of incubation, and also was proportional to the amount of cellular protein. Similar results were obtained for leukocytes. Table 1 shows control values for the pyruvate oxidation rate in leukocytes and fibroblasts. The CV for the assay amounts to 7%, both for leukocytes and fibroblasts. The Lineweaver–Burk plots with [1-14C]-pyruvate as a substrate revealed an apparent K_m value...
Table 1. Pyruvate Oxidation in Human Leukocytes and Cultured Fibroblasts

<table>
<thead>
<tr>
<th></th>
<th>[1-14C]Pyruvate</th>
<th>[2-14C]Pyruvate</th>
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<tbody>
<tr>
<td><strong>Leukocytes</strong></td>
<td></td>
<td></td>
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<tr>
<td>2.8 ± 0.9</td>
<td>2.1 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>range 1.4–4.1 (n = 11)</td>
<td>range 1.1–3.5 (n = 12)</td>
<td></td>
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<tr>
<td><strong>Fibroblasts</strong></td>
<td></td>
<td></td>
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<tr>
<td>37 ± 14</td>
<td>18 ± 7</td>
<td></td>
</tr>
<tr>
<td>range 19–61 (n = 10)</td>
<td>range 12–30 (n = 5)</td>
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*a* Values are given as the mean ± SD.

*b* nmol/h per 10⁶ leukocytes.

*c* nmol/h per mg of protein.

Fig. 1. Oxidation rates of [1-14C]pyruvate and [2-14C]pyruvate in intact cultured fibroblasts incubated for various periods of time (using 1.0 mg of protein) and with increasing enzyme concentrations (incubation time, 1 h).

for leukocytes of 0.14 mmol/liter and for fibroblasts of 0.11 mmol/liter.

Determination of citric acid cycle activity in intact leukocytes and fibroblasts with its intermediates as substrates was almost impossible. Citric acid cycle activity could be only demonstrated by using [2-14C]pyruvate as substrate (Table 1). No measurable activity was found with [U-14C]malate, [1.5-14C]- and [6-14C]citrate, [1.4-14C]succinate, or [5-14C]2-oxoglutarate as substrates. The oxidation rate with [1-14C]2-oxoglutarate and [1-14C]glutamate as substrates was low in intact cells.

Table 2 compares the oxidation rates of [1-14C]pyruvate and [1-14C]2-oxoglutarate by intact leukocytes and fibroblasts and by their mitochondria. Cytochrome c oxidase and glutamate dehydrogenase were measured as mitochondrial markers. The ratio of pyruvate dehydrogenase activity to activity of mitochondrial markers is in the same order of magnitude for intact cells and mitochondria. The discrepancy of the ratio for pyruvate dehydrogenase to glutamate dehydrogenase between intact cells and mitochondria might be due to leakage of glutamate dehydrogenase out of the mitochondrial matrix. The ratio for 2-oxoglutarate dehydrogenase activity was, however, much higher in mitochondria than in intact cells. Cytochrome c oxidase activity in leukocytes was too low to be used as a reliable marker.

**Discussion**

Several clinical disorders are associated with increased concentrations of lactate or pyruvate (or both) in blood or cerebrospinal fluid (or both). The mean clinical features of patients suffering from these disorders are mental retardation, muscular hypotonia, and convulsions (1). Measuring pyruvate oxidation rate in leukocytes and fibroblasts can be helpful in the diagnosis of children suffering from lactic acidemia.

Leukocytes and fibroblasts contain the enzymatic equipment for pyruvate oxidation (9–11), such as pyruvate dehydrogenase (1), the citric acid cycle (12), and the electron transport system (13–15).

The oxidation rates of [1-14C]pyruvate and [2-14C]pyruvate we found for intact leukocytes and fibroblasts (Table 1) much exceed those previously published (1, 16, 17), perhaps because of the higher substrate concentration we used. In other investigations (1, 16, 17) pyruvate concentration was below the Kₘ value.

Pyruvate is oxidized in fibroblasts more rapidly than

Table 2. Pyruvate Dehydrogenase and 2-Oxoglutarate Dehydrogenase Activity in Intact Cells and Mitochondria of Fibroblasts and Leukocytes.

<table>
<thead>
<tr>
<th></th>
<th>Fibroblasts</th>
<th>Mitochondria</th>
<th>Leukocytes</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact cells</td>
<td></td>
<td>Intact cells</td>
<td>Mitochondria</td>
</tr>
<tr>
<td><strong>Activity (nmol/h per mg of protein)</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Pyruvate dehydrogenase</td>
<td>38</td>
<td>218</td>
<td>2.9</td>
<td>16.6</td>
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<tr>
<td>2-Oxoglutarate dehydrogenase</td>
<td>5</td>
<td>77</td>
<td>0.2</td>
<td>10.7</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>1242</td>
<td>6930</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Glutamate dehydrogenase</td>
<td>3060</td>
<td>11 820</td>
<td>492</td>
<td>1270</td>
</tr>
<tr>
<td><strong>Ratio (× 10⁴)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate dehydrogenase</td>
<td>30.6</td>
<td>31.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>4.0</td>
<td>11.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2-Oxoglutarate dehydrogenase</td>
<td>12.4</td>
<td>18.4</td>
<td>5.9</td>
<td>13.0</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>1.6</td>
<td>6.5</td>
<td>0.5</td>
<td>8.4</td>
</tr>
</tbody>
</table>

in leukocytes. The oxidative pathway in leukocytes is of minor importance (18). The generation of ATP quantitatively depends less on oxidative phosphorylation than on glycolysis (7).

The approximately equal $^{14}$CO$_2$ production from $[1-^{14}$C]$\text{pyruvate}$ and $[2-^{14}$C]$\text{pyruvate}$ with intact leukocytes and fibroblasts (Table 1) contrasts with the results presented by Cederbaum et al. (16) and with the observations on rat adipose tissue (19). Our data suggest that in leukocytes and fibroblasts acetyl-CoA produced from pyruvate is almost completely oxidized in the citric acid cycle under the conditions we used.

No reliable measurements of citric acid cycle activity could be performed with intermediates of the citric acid cycle as substrates in intact leukocytes and fibroblasts. This might be due to a low activity of the citric acid cycle or to the limited transport of these substrates across the cell membrane or the mitochondrial membrane. The experiments with $[2-^{14}$C]$\text{pyruvate}$ as substrate show that in vitro measurement of the overall citric acid cycle activity is feasible. Moreover, earlier studies (9, 12) demonstrated a definite citric acid cycle activity in both types of cells. The low rate of transport of 2-oxoglutarate across the cell membrane will cause a difference in its rate of oxidation between intact cells and mitochondria.

We conclude that pyruvate oxidation can be reliably measured in intact leukocytes and fibroblasts with use of $[1-^{14}$C]$\text{pyruvate}$. Concomitant assay of $^{14}$CO$_2$ production from $[2-^{14}$C]$\text{pyruvate}$ can provide an indication for the total activity of the citric acid cycle, but the individual steps of this cycle can only be determined by using mitochondria or disrupted cells. Prolonged trypsinization of fibroblasts may increase the cellular uptake of di- and tricarboxylic acids (12) but may disturb cellular metabolism. We suggest that use of intact leukocytes, and especially fibroblasts, may be appropriate to detect defects in pyruvate oxidation clinically.

References