Is absence of pyruvate dehydrogenase complex in mitochondria a possible explanation of significant aerobic glycolysis by normal human leukocytes?

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Abstract The oxygen consumption of leukocyte mitochondria of both healthy donors and leukemic patients was tested by using different respiratory substrates. The results indicate that pyruvate could not be utilized by mitochondria of normal leukocytes, whereas mitochondria of leukemic leukocytes could use pyruvate as a good respiratory substrate. A search for the possible presence of pyruvate dehydrogenase complex (PDC) in leukocytes indicates that this enzyme is apparently absent in mitochondria of normal leukocytes but is quite active in mitochondria of leukemic leukocytes. The absence of PDC in normal leukocyte mitochondria can explain the phenomenon of significant aerobic glycolysis that has been observed in normal leukocytes.

Key words: Leukocyte mitochondrion; Pyruvate dehydrogenase complex; Aerobic glycolysis; Leukemia

1. Introduction

It has been known for a long time that malignant cells produce lactic acid from glucose at an abnormally high rate even in the presence of oxygen which normally inhibits this process [1,2]. However, several normal tissues such as retina, intestinal mucosa, kidney medulla [3], and normal leukocytes [4] also exhibit a similar phenomenon. We have recently studied the effect of methylglyoxal, a potent anticancer agent, on the mitochondrial respiration of human leukocytes, both normal and malignant, with different respiratory substrates [5]. During the course of this study, we made an interesting observation: pyruvate, which is a usual substrate of mitochondrial respiration, cannot be utilized by mitochondria of normal leukocytes, whereas pyruvate as usual is oxidized by mitochondria of leukemic leukocytes. On further investigation, we observed that PDC, the enzyme necessary for the oxidation of pyruvate, is absent in the mitochondria of normal leukocytes. This absence of PDC in normal leukocytes can explain the phenomenon of significant aerobic glycolysis in these cells.

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Abbreviations: PDC, pyruvate dehydrogenase complex

2. Materials and methods

2.1. Chemicals and blood

All the biochemicals and the mitochondrial respiratory inhibitors rotenone and malonate were purchased from Sigma Chemical Co., St. Louis, MO, USA. Other chemicals were of analytical grade and were obtained from local manufacturers.

Blood of leukemic patients (suffering from chronic myeloid leukemia or acute myeloid leukemia or chronic lymphocytic leukemia or acute lymphocytic leukemia) were collected from the hematology unit of the hospital. Patients with chronic myeloid leukemia were selected for this study if their peripheral leukocyte count ranged from 90 000 to 150 000 cells/ml with almost 95% of these cells of the leukemic series. For the other types of leukemia, patients were selected with leukocyte counts of more than 25 000 cells/ml. Normal blood was from healthy donors. For collection of blood, informed consent was obtained from the donors. The blood was brought to the laboratory at ambient temperature and the experiments were started within 1 h after withdrawal of blood.

For respiratory studies, blood was collected from 10 healthy male donors (26–40 years old) and nine male and five female patients (9–33 years old). For the assay of PDC in leukocyte preparations, blood was collected from 10 healthy male donors (25–45 years old) and five male and one female patients (18–50 years old). We have observed that age and sex of donors have no influence on the experimental results. Results presented in this paper for leukemic blood were obtained from leukocytes of patients with chronic myeloid leukemia. Similar results were also obtained with other types of leukemic leukocytes.

2.2. Preparation and assay of enzymes

Leukocytes were isolated from whole blood and mitochondria were prepared and the respiration was measured as described previously [5]. The mitochondrial suspensions were sonicated for a total period of 2 min (30 s each, 4 times), and centrifuged at $30\,000\times g$ for 30 min. After discarding the precipitate, the supernatant (approximately 2.3 ml from 50 ml of whole blood) was used for PDC activity.

2.3. PDC from goat heart

All operations were at 0–4°C. 5 g of goat heart tissue obtained from a local slaughterhouse was homogenized in a loosely fitting Potter-Elvehjem homogenizer with 15 ml of 0.25 sucrose, 25 mM potassium phosphate buffer, pH 7.4 and 0.2 mM EDTA. The homogenate was centrifuged at $2000\times g$ for 10 min and the pellet was discarded. The supernatant was centrifuged at $22\,000\times g$ for 10 min and the supernatant was discarded. The pellet was suspended in 0.25 M sucrose containing 25 mM potassium phosphate buffer pH 7.4 and centrifuged at $30\,000\times g$ for 10 min. The precipitate was washed twice and was suspended in the same buffer and sonicated for 2 min (30 s each, 4 times). The sonicated material was centrifuged at $30\,000\times g$ for 10 min and after discarding the precipitate, the supernatant (approximately 2.5 ml) was used for PDC activity.

2.4. Arylamine acetyltransferase

This enzyme was partially purified from pigeon liver. All operations were at 0–4°C. 2 g of pigeon liver was homogenized in a Potter-Elvehjem homogenizer with six volumes of 25 mM sodium phosphate buffer, pH 7.4 containing 2 mM β-mercaptoethanol. The homogenate was centrifuged at $10\,000\times g$ for 10 min and the precipitate was dis-

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carded. To the supernatant (10 ml), 1 ml of 1% protamine sulfate was added dropwise. After standing for 10 min, the suspension was centrifuged as above and the precipitate was discarded. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation. The enzyme fraction which appeared at 40–60% $(NH_4)_2SO_4$ saturation was subjected to dialysis against 25 mM Na-phosphate buffer, pH 7.4 containing 2 mM β-mercaptoethanol. This enzyme preparation which was free of any PDC contamination was used as arylamine acetyltransferase activity.

2.5. Assay of enzymes and estimation of protein

The assay of PDC was done according to the method of Coore et al. with minor modifications [6]. The enzyme was assayed by coupling with arylamine acetyltransferase [7] and monitoring the change in extinction at 420 nm due to acetylation of *p*-nitroaniline by the acetyl-CoA formed in the PDC reaction. The assay mixture contained in a total volume of 1 ml, 50 μmol of sodium phosphate buffer, pH 7.9, 1 μmol of NAD, 0.01 μmol of thiamine pyrophosphate, 1 μmol of $MgCl_2$, 2 μmol of pyruvate, 0.16 μmol of *p*-nitroaniline, 0.1 unit of arylamine acetyltransferase; the reaction was started by the addition of 0.2 μmol of coenzyme A. Appropriate control was made with boiled PDC or omitting coenzyme A in the incubation mixture. We could not assay PDC activity by monitoring the formation of NADH due to the presence of significant NADH oxidizing activity in our enzyme preparation.

One unit activity of PDC is defined as the amount of the enzyme required to cause a decrease in absorbance of 0.57 due to acetylation of 0.1 μmol of *p*-nitroaniline. Specific activity is defined as units of activity per mg of protein.

The assay mixture for arylamine acetyltransferase contained in a total volume of 1 ml, 100 μmol of sodium phosphate buffer, pH 6.8, 0.16 μmol of *p*-nitroaniline, 0.15 μmol of acetyl-CoA and the requisite amount of the enzyme. The decrease in absorbance at 420 nm was monitored. The absorbance of 0.1 μmol of *p*-nitroaniline at 420 nm is 0.570. One unit of arylamine acetyltransferase activity was defined as the amount of the enzyme required to acetylate 1 μmol of *p*-nitroaniline per minute under the standard assay conditions.

Protein was estimated with bovine serum albumin as standard by the method of Lowry et al. [8].

3. Results

3.1. Oxygen uptake by mitochondria of normal leukocytes with different respiratory substrates

In Fig. 1, trace a shows that malate plus pyruvate, which are typical respiratory substrates for other normal cellular mitochondria, could not be used by normal leukocyte mitochondria. But when NAD was added to the system, a significant respiratory rate was observed. Moreover, normal leukocyte mitochondria consumed oxygen at a significant rate with only malate in the presence of NAD and pyruvate had no effect on the rate of oxygen uptake with malate and NAD as respiratory substrates (trace b). Trace c shows that in the absence of malate, pyruvate could not be used as respiratory substrate even in the presence of NAD, but when malate was added to the system a rapid rate of oxidation began, and this respiration could as usual be inhibited by the mitochondrial complex I-specific inhibitor rotenone. Succinate could elicit a moderately higher rate of respiration by normal leukocyte mitochondria in the absence of NAD and this succinate-dependent respiration could as usual be inhibited by malonate (trace d). Moreover, NAD has no effect on succinate-dependent respiration (data not presented).

All these results strongly suggest that pyruvate could not be utilized by normal leukocyte mitochondria.

3.2. Oxygen consumption of mitochondria of leukemic leukocyte with various respiratory substrates

In Fig. 2, trace a shows that, in contrast to normal leukocyte mitochondria, mitochondria of leukemic leukocytes could

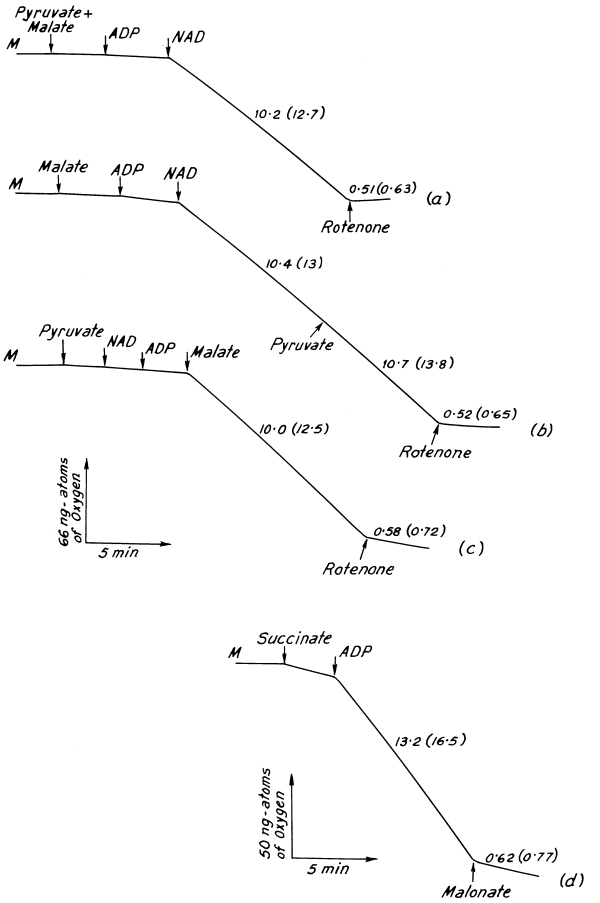

Fig. 1. Oxygen uptake by mitochondria (M) of normal leukocytes with different respiratory substrates. The direct oxygraph tracings of a typical experiment are shown. Addition of different substrates and inhibitors is indicated in the respective tracings. The numbers outside parentheses and the numbers within parentheses along the tracings represent the rate of oxygen consumption (ng atoms of oxygen/min and ng atoms of oxygen/min/mg of protein rerspectively). In each case, in a control set of experiments in which no inhibitor was added, mitochondria can consume oxygen at a linear rate for a period of at least 30 min. The total amounts of oxygen and mitochondrial protein present in the incubation mixture were approximately 990 ng atoms and 0.8 mg respectively.

oxidize pyruvate plus malate at a fairly high rate even in the absence of NAD, and NAD has a little stimulatory effect on this malate plus pyruvate-dependent respiration. Malate in the presence of NAD could also be oxidized, but pyruvate had a substantial stimulatory effect (trace b). Trace c shows that pyruvate in the presence of NAD could not elicit any respiratory rate, but when malate was added to the system, a rapid rate of oxygen consumption was observed. The FAD-linked substrates succinate (trace d) and α-glycerophosphate (not presented) could be oxidized at a much faster rate and the oxidation of succinate could be conveniently blocked by malonate, a known inhibitor of succinic dehydrogenase (complex II). The respiration of leukemic leukocyte mitochondria with α-oxoglutarate, succinate and α-glycerophosphate was not affected by NAD (not presented).

3.3. The status of PDC in normal and leukemic leukocyte mitochondria

All the results presented above strongly suggest that pyru-

vate could not be oxidized by mitochondria of normal leukocytes, whereas mitochondria of leukemic leukocytes, like the mitochondria of other normal cells, could use pyruvate as a respiratory substrate. This interesting finding prompted us to investigate the status of PDC in both normal and leukemic leukocyte mitochondria. It is well known that pyruvate generated in glycolysis is transported into mitochondria and is oxidized through the citric acid cycle after being converted to acetyl-CoA by PDC.

The results of our investigation indicate that pyruvate could not be converted to acetyl-CoA by a crude mitochondrial preparation of normal leukocytes, whereas a mitochondrial preparation of leukemic leukocytes could convert pyruvate to acetyl-CoA at a substantial rate and this rate is comparable with the activity of PDC present in the crude preparation of goat heart mitochondria (Fig. 3).

It is well known that phosphorylation of the pyruvate dehydrogenase component of PDC inactivates the enzyme complex and dephosphorylation reactivates the complex [9]. The phosphorylated (inactive) enzyme complex is reactivated in the presence of Mg^{2+} [10]. However, we could not obtain PDC activity in normal leukocyte mitochondrial preparations even after incubation of these preparations with Mg^{2+}.

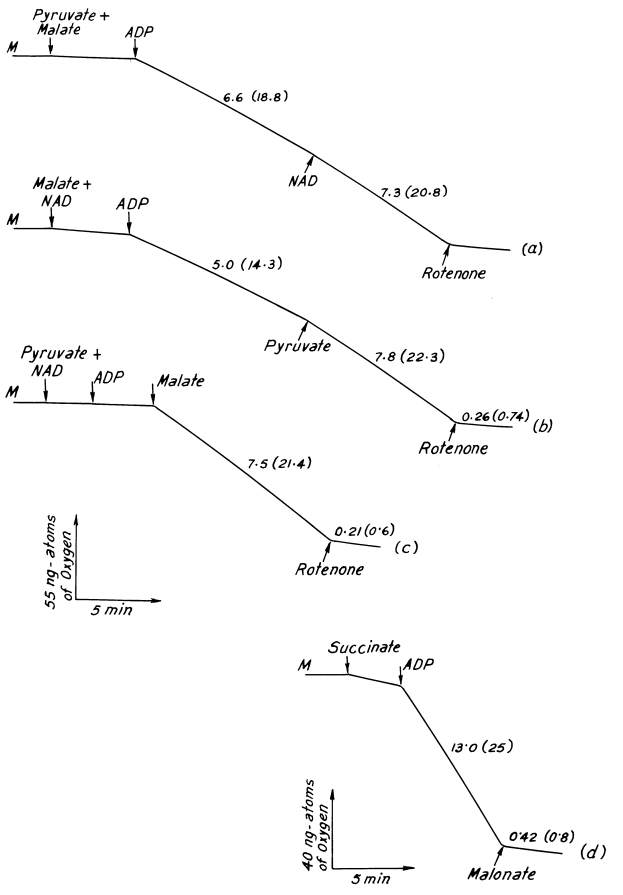

Fig. 2. Oxygen uptake by mitochondria of leukemic leukocytes with different respiratory substrates. The details are similar to the legends of Fig. 1 except that the incubation mixtures contained approximately 0.35 mg of mitochondrial protein for tracings a, b and c and 0.5 mg of mitochondrial protein for tracing d.

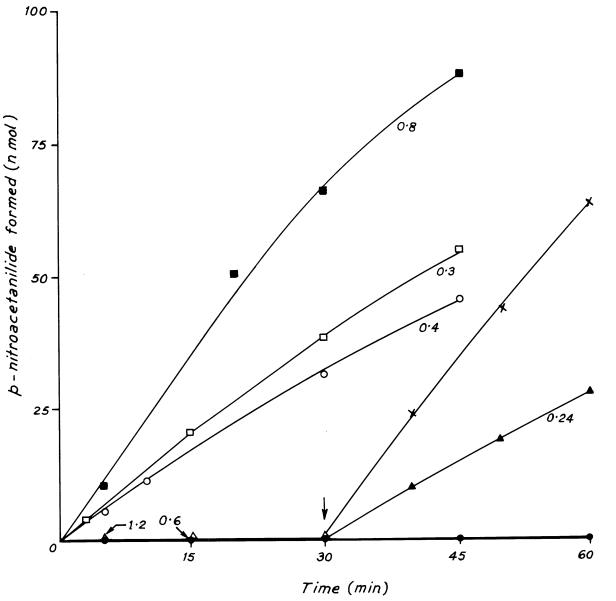

Fig. 3. Formation of *p*-nitroacetanilide by PDC- or acetyl-CoA-dependent acetylation of *p*-nitroaniline. ○ and ■ represent acetylation by a crude PDC preparation from leukemic leukocytes; ● and △ that by a crude preparation from leukocytes isolated from the blood of healthy donors; □ that by PDC partially purified from goat heart; formation of *p*-nitroacetanilide by the addition (indicated by ↓) of either crude PDC from goat heart or known acetyl-CoA (0.1 μmol) to the incubation mixture containing crude preparation from normal leukocytes is represented by ▲ and × respectively. The numbers on the curve represent mg of protein used in the assay mixtures.

4. Discussion

As mentioned before, rapidly growing malignant cells have a high rate of aerobic glycolysis and this phenomenon has been considered by many biochemists to be a fundamental feature of malignant aberration. However, it has also been observed that several non-cancerous tissues such as retina, normal leukocytes, kidney medulla, etc. have appreciable aerobic glycolysis [3,4]. Several attempts have been made to provide an explanation for the aerobic glycolysis observed in these cells. The results presented in this paper convincingly demonstrate that the enzyme PDC is absent in normal leukocytes but is present in leukemic leukocytes. Due to the absence of PDC in normal leukocytes, the pyruvate that is generated in the glycolytic pathway cannot enter into the citric acid cycle. As a result, the active L-lactic dehydrogenase which is present in the cellular pool converts pyruvate to L-lactic acid resulting in appreciable aerobic glycolysis. The present study, however, has not investigated the status of PDC in different varieties of normal leukocytes. But we can assume that the absence of PDC in neutrophils and also possibly in lymphocytes can account for the appreciable lactic acid formation in total leukocytes, considering the relative number of these cells present.

One peculiar property of normal leukocyte mitochondria is that they cannot oxidize malate without the addition of NAD [11]. Like the previous work of Foster and Terry [11] with normal human leukocyte mitochondria, we observed that the rate of oxidation with only malate was very feeble, but when NAD was added to the incubation mixture the rate was

increased substantially. In several previous studies on the oxidation of different substrates by plant mitochondria, NAD had been added in the incubation mixture which resulted in an appreciable increase in the rate of oxidation of these substrates [12,13].

The results presented in this paper also illuminate the interrelationship of two energy (ATP) producing processes, namely glycolysis and mitochondrial respiration, in normal and malignant cells. In several recent publications from our laboratory, we have provided strong experimental evidence and theoretical arguments which suggest that excessive ATP formation in cells may lead to malignancy [5,14,15]. In contrast to the established idea, in malignant cells, not only glycolysis is enhanced, but mitochondrial respiration is also elevated so as to favor excessive ATP formation. From the results presented in this paper we can assume that in normal leukocytes the gene responsible for PDC is somehow not expressed, whereas during the process of malignant transformation the PDC gene is expressed and the enzyme is synthesized. The presence of this enzyme in leukemic leukocytes further strengthens the oxidative machinery of the cells.

The multienzyme PDC has been purified from different sources and the mechanism of interaction of different enzyme components has been studied to understand the regulation of flux of pyruvate through this enzyme complex. It is generally assumed that the PDC reaction is controlled mainly by a highly regulated phosphorylation/dephosphorylation cycle (see [9,10,16] and the references cited therein). But our studies have implicated this enzyme complex in malignant aberrations. It will be interesting to study the expression of the PDC gene during malignant transformation at least in human leukocytes.

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