Carrier Detection of Pyruvate Carboxylase Deficiency in Fibroblasts and Lymphocytes

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Summary

Pyruvate carboxylase (E.C. 6.4.1.1) activity was determined in the circulating peripheral lymphocytes and cultured skin fibroblasts from the family of a patient with hepatic, cerebral, renal cortical, leukocyte, and fibroblast pyruvate carboxylase deficiency (PC^{Portland} deficiency). Lymphocyte activities were: mother, 33-39%; father, 11-29%; brother, 82-103%; and sister, 38-48% of the lowest normal. Fibroblasts from the patient's mother and father had 42 and 34%, respectively, of the activity of the lowest normal. These data demonstrate that the disease is inherited in an autosomal recessive manner and that lymphocytes and fibroblasts can be used to detect carriers. Neither pyruvate carboxylase nor mitochondrial PEPCK activity in lymphocytes was increased by a 21-hr fast.

Speculation

The demonstration that carriers of pyruvate carboxylase deficiency can be detected with a venous blood sample may allow, with the use of extensive family studies, the assignment of this enzyme to a chromosomal linkage group.

Pyruvate carboxylase deficiency (2, 3, 6, 7), an inborn error of pyruvate metabolism, usually results in severe developmental and mental retardation, lactic acidosis and, in at least one case (2, 3) proximal renal tubular acidosis. Until recently, diagnosis of the disease has been possible only from a liver biopsy and this has precluded family studies and prenatal diagnosis. However, the recent development by Atkin *et al.* (4) of an assay for pyruvate carboxylase in cultured skin fibroblasts and circulating peripheral lymphocytes has allowed for the first time the detection of pyruvate carboxylase deficiency in the leukocytes and fibroblasts of a patient with hepatic, renal cortical, and cerebral pyruvate carboxylase deficiency. DeVivo *et al.* (6) also demonstrated pyruvate carboxylase deficiency in the fibroblasts of a patient with hepatic pyruvate carboxylase deficiency (PC^{St. Louis} deficiency) and reduced pyruvate carboxylase activity in fibroblasts from that patient's parents. In the present study, examination of fibroblasts and lymphocytes from the parents of the patient with PC^{Portland} deficiency and of lymphocytes from the patient's two normal siblings establish the mode of inheritance of pyruvate carboxylase deficiency. Furthermore, this study demonstrates that either fibroblasts or lymphocytes may be useful in the detection of carriers of pyruvate carboxylase deficiency.

MATERIALS AND METHODS

Fibroblasts were grown in Eagle's MEM with 10% fetal bovine serum, 1000 units/ml penicillin G and 50 µg/ml gentamycin, fed every 2-3 days and harvested, as described (4), 3-14 days past confluency. The control donors varied from <3 days to 45 yr of age and included both sexes. All tests of fibroblasts for myco-

plasma (12) and bacteria were negative. The lymphocyte plus monocyte or mixed leukocyte fractions were isolated as described (4) from whole venous blood. Care was taken to eliminate donors that were obese, malnourished, had diabetes mellitus or a family history of the same, were on "unusual" diets or were receiving any medication. Some of the women were, however, taking birth control pills. The donors were instructed that the meal to be eaten before venipuncture should be "balanced" with respect to protein, fat, and carbohydrate. Most donors were assayed for mitochondrial phosphoenolpyruvate carboxykinase (E.C.4.1.1.32, PEPCK) and citrate synthase (E.C.4.1.3.7) in addition to pyruvate carboxylase. Two controls of the same sex and approximate age (controls were about 1 yr older than the patient) as the patient were tested. Pyruvate carboxylase and mitochondrial PEPCK were assayed by a modification (4) of the methods of Utter and Keech (13) and Ballard and Hanson (5). Citrate synthase was assayed as described by Isohashi *et al.* (8) by a spectrophotometric assay which uses 3,5'-dithiobis(2-nitrobenzoate) to measure the release of free CoA-SH. Protein was determined by the method of Lowry *et al.* (9). All reagents were of the grade and source designated in the earlier publication (4). All blood samplings and assays of suspected carriers of, or patients with pyruvate carboxylase deficiency were accompanied by the simultaneous sampling and assay of a known normal. All skin biopsies and blood samples were obtained with informed consent.

RESULTS

The data in Table 1 demonstrate that fasting for 4, 8, or 21 hr had no significant effect on lymphocyte pyruvate carboxylase activity when blood was drawn at the same time of day (3:30-4:00 PM). However, the mean and range of lymphocyte pyruvate carboxylase activities of 7 samples drawn at 9 AM (15 hr postprandial) were appreciably lower than the mean and range of samples drawn at 3:30-4:00 PM, whether 4, 8, or 21 hr postprandial. Lymphocyte mitochondrial PEPCK activities in blood drawn at 9 AM (15 hr postprandial) were also lower than those drawn at 3:30-4:00 PM (4 hr postprandial) (Table 1). Neither fasting nor time of day had any consistent effect on the activity of lymphocyte citrate synthase (Table 1). Pyruvate carboxylase activity in the mixed leukocytes of three normal adults was 0.089 ± 0.012 munits/mg mononuclear leukocyte protein and in two normal children (5 yr old) was 0.089 ± 0.005 (0.084, 0.094) munits/mg mononuclear leukocyte protein. All the above had leukocytes drawn at 3-4 PM when individuals were about 3½ hr postprandial. Two normal adults whose blood was drawn at 9:30 AM (15 hr postprandial) had values of 0.047 and 0.042 munits/mg mononuclear leukocyte protein. One of these morning donors had a value of 0.070 munits/mg mononuclear leukocyte protein when blood was drawn in the afternoon.

Lymphocyte pyruvate carboxylase activity for normals, the patient's parents and brother and sister are shown in Figures 1 and 2. Pyruvate carboxylase activity in cultured skin fibroblasts

Table 1. Effect of fasting and time of day on human lymphocyte pyruvate carboxylase, PEPCK, and citrate synthase activities

Time of day	Pyruvate carboxylase mUnits/mg protein ¹	PEPCK mUnits/mg protein ¹	Citrate synthase mUnits/mg protein ¹
9 AM, 14–15 hr postprandial	0.104 ± 0.022 (n = 7) (0.037–0.185)	0.89 ± 0.117 (n = 6) (0.311–1.12)	67.7 ± 15.0 (n = 7) (22.8–128)
3:30–4:00 PM, 3½–4 hr postprandial	0.150 ± 0.017 (n = 8) (0.092–0.262)	1.42 ± 0.119 (n = 6) (0.824–1.49)	66.4 ± 6.3 (n = 6) (46.3–95.7)
3:30–4:00 PM, 8 hr postprandial	0.149 ± 0.025 (n = 4) (0.96–0.203)		85.2 ± 6.2 (n = 4) (75.7–102)
3:30–4:00 PM, 20–21 hr postprandial	0.167 ± 0.019 (n = 7) (0.098–0.260)		77.7 ± 6.3 (n = 7) (58.2–109)

¹ mUnits enzyme activity for pyruvate carboxylase and PEPCK: nmoles HCO₃ fixed/min, citrate synthase: nmoles CoA-SH produced/min. All assays at 37°C. Mg protein: mg lymphocyte plus monocyte protein. Values are expressed as the mean ± SEM (11) followed by the range and number of control subjects (n).

Fig. 1. Pyruvate carboxylase activity in lymphocytes from normals and family of patient with PC^{Portland} deficiency. The values for the patient's family represent the mean of two separate assays on one blood sample with the range designated by bars. One mUnits equals nmoles HCO₃ fixed/min at 37°C. The following symbols are used: normals (○); assays on five different blood samples drawn from the same normal subject over the period of a year (Δ); female carrier of pyruvate carboxylase deficiency (◐); male carrier of pyruvate carboxylase deficiency (◑); male examined for pyruvate carboxylase deficiency and found to be normal (◒); and patient with pyruvate carboxylase deficiency (■). The dotted line indicates 50% of the activity of the lowest normal. The mean of normals is marked by an arrow. The normals consist of 11 different individuals some of whom had blood drawn at different times postprandial. However, all blood was drawn from 3:30–4:00 PM.

from normals, the patient, and the patient's parents is shown in Figures 2 and 3. The activity of mitochondrial PEPCK and citrate synthase in the lymphocytes and fibroblasts of the family members is given in Table 2.

The activity of pyruvate carboxylase in the fibroblasts of the patient of DeVivo *et al.* (6) with PC^{St. Louis} deficiency and of that patient's parents is given in Figure 3. The activities of mitochondrial PEPCK and citrate synthase in these fibroblasts are given in Table 2.

DISCUSSION

It was reasonable to predict that pyruvate carboxylase, an essential enzyme for gluconeogenesis in mammalian liver and renal cortex, might exhibit elevated activity during physiologic conditions, such as fasting, that demand increased gluconeogenesis

Fig. 2. Pedigree of family with PC^{Portland} deficiency. The proband, II 3, died at 46 months of age. Values represent nmoles HCO₃ fixed/min/mg protein at 37°C. Fblst: activity determined in fibroblasts; lymph: activity determined in lymphocytes; leuk: activity determined in a mixed leukocyte preparation but corrected for mononuclear leukocyte content and activity expressed in terms of mg of mononuclear leukocyte protein. Normal values: fibroblasts, 1.35 ± 0.173 (0.95–1.85) (n = 5); lymphocytes, 0.156 ± 0.011 (0.096–0.262) (n = 19); leukocytes, 0.089 ± 0.013 (0.070–0.110) (n = 5) mUnits/mg protein.

and that it might be responsive to one or more of those hormones regulating glucose homeostasis: insulin, glucagon, and catecholamines. There are conflicting reports with regard to this enzyme's response to these conditions, but the question must still be considered open. Also, the blood samples for pyruvate carboxylase were to be assayed for PEPCK and at the time of the initial blood samples, it was not known whether the lymphocyte PEPCK activity was due to the cytosolic or mitochondrial form of this enzyme and it had been well established that cytosolic hepatic PEPCK activity does increase in response to fasting (10). Furthermore, lymphocyte pyruvate carboxylase, other than the studies of Atkins *et al.* (4), had never before been assayed and consequently the influence, if any, of different physiologic states such as fasting, or endogenous rhythms on the enzyme's activity was unknown. Initially, therefore, all blood samples were drawn either at 9:30 AM or 3–4 PM to reduce the possible effects of varying physiologic states on lymphocyte pyruvate carboxylase and PEPCK activity and care was taken to match the postprandial intervals for experimental and control subjects.

There was no significant difference in the mean or range of

pyruvate carboxylase activities obtained 4, 8, or 21 hr postprandial if the blood was drawn at 3:30–4:00 PM. There was, however, a possible effect of the time of day at which blood was drawn, on pyruvate carboxylase and PEPCK activity (Table 1). Citrate syn-

Fig. 3. Pyruvate carboxylase activity in fibroblasts from normals, obligate carriers, and patients with pyruvate carboxylase deficiency. Values for carriers represent the mean of two separate assays with the range designated by bars. One mUnits equals nmoles HCO_3^- fixed/min at 37°C . The origin of fibroblast lines is designated in Table 3 of Atkin *et al.* (4). Fibroblasts were grown, harvested, and assayed as described in the *Methods* section. The dashed line marks 50% of the lowest normal. The arrow marks the mean of the normals. Symbols: Mother ($\bullet^{\text{St. L.}}$), father ($\bullet^{\text{St. L.}}$), and patient ($\bullet^{\text{St. L.}}$) with $\text{PC}^{\text{St. Louis}}$ deficiency; mother ($\bullet^{\text{P}}$), father ($\bullet^{\text{P}}$), and patient ($\bullet^{\text{P}}$) with $\text{PC}^{\text{Portland}}$ deficiency.

thase exhibited no significant effect although the large variation in the activity of this enzyme may have obscured any trend. The possible existence of diurnal variation in pyruvate carboxylase activity is given support by the finding that the activity was higher in the leukocytes of five normals drawn in the afternoon than that of two normals drawn in the morning (See *Results*). Furthermore, these data contain one individual tested both in the afternoon and morning who exhibited decreased pyruvate carboxylase activity in the morning sample. The physiologic significance of this observation is different to evaluate. It is possible that the pyruvate carboxylase and PEPCK activities are actually lower in the morning as a result of an effect on enzyme activity by a diurnal hormonal rhythm(s). It is also possible that the population of lymphocytes plus monocytes is different in the morning than the afternoon. No attempt was made in this study to determine whether the pyruvate carboxylase and PEPCK activities are distributed equally among monocytes, T, and B lymphocytes. Thus, the difference between morning and afternoon activities could reflect a difference in cell population.

Because of the possible difference between morning and afternoon samples, only those determinations made on lymphocytes drawn in the afternoon were used to establish the normal range. The practical necessity of drawing the experimental and control blood samples at the same time of day is underscored by the finding of lymphocyte pyruvate carboxylase activities, from some presumed normals sampled in the morning, in the carrier range if the normal range were established with afternoon samples. Afternoon sampling of subjects has the advantage, furthermore, of providing a greater yield of white cells and, thus, permitting the drawing of a smaller blood sample. This is not a minor consideration when testing pediatric patients because the pyruvate carboxylase activity in the lymphocytes of a carrier is vanishingly small.

Although the patient's brother's two lymphocyte pyruvate carboxylase activities (0.099 and 0.079) were below the 68.3% confidence interval for normals, the existence of two normal values (0.096 and 0.098) (Fig. 1) below the patient's brother's higher value, make it unlikely that the brother is a carrier for pyruvate carboxylase deficiency. The sister, mother, and father with lymphocyte pyruvate carboxylase activities of 38–48, 33–39, and 11–29%, respectively, of the lowest normal, clearly are carriers for pyruvate carboxylase deficiency (Figs. 1 and 2). Although the

Table 2. Activities of mitochondrial PEPCK and citrate synthase in lymphocytes and fibroblasts of two families with pyruvate carboxylase deficiency

Subject	PEPCK mU/mg protein ¹	Citrate synthase mU/mg protein ¹
Lymphocytes		
Normals	1.31 $\pm$ 0.112 (<i>n</i> = 8) (0.824–1.88)	75.1 $\pm$ 3.72 (<i>n</i> = 18) (50.5–109)
Pyruvate carboxylase ^{Portland} deficiency		
Father	2.95 $\pm$ 0.107 (1)	67.9 $\pm$ 5.9 (2)
Mother	0.884 $\pm$ 0.049 (1)	55.5 $\pm$ 8.9 (2)
Sister	1.15 $\pm$ 0.013 (1)	50.7 $\pm$ 16.1 (2)
Brother	1.92 $\pm$ 0.010 (1)	58.5 $\pm$ 2.5 (2)
Patient ²	0.930 $\pm$ 0.13 (1)	47.6 $\pm$ 0.8 (1)
Fibroblast		
Normals	5.57 $\pm$ 0.32 (<i>n</i> = 3) (4.56–6.43)	42.97 $\pm$ 9.13 (<i>n</i> = 3) (13.3–73.7)
Pyruvate carboxylase ^{Portland} deficiency		
Father	not determined	70.8 $\pm$ 6.7 (2)
Mother	5.94 $\pm$ 0.061 (1)	37.1 $\pm$ 8.4 (3)
Patient	0.954 $\pm$ 0.134 (2)	28.9 $\pm$ 2.2 (4)
Pyruvate carboxylase ^{St. Louis} deficiency		
Father	3.94 $\pm$ 0.052 (1)	26.0 $\pm$ 9.7 (2)
Mother	6.33 $\pm$ 0.33 (2)	29.3 $\pm$ 10.9 (2)
Patient	3.36 $\pm$ 0.071 (1)	36.6 $\pm$ 8.6 (3)

¹ mUnits of enzyme activity as described in the legend of Table 1. Value are expressed as the mean $\pm$ SEM (11) for the number of assays on that tissue in parentheses. If the tissue was only assayed once, the value represents the mean $\pm$ SEM of the replicates of the assay from which replicate blanks were subtracted. The number of replicates for pyruvate carboxylase or PEPCK in lymphocytes or leukocytes was 6, for pyruvate carboxylase and PEPCK in fibroblasts, 4 and for citrate synthase, 2.

² Assayed in mixed peripheral leukocytes, but corrected for mononuclear leukocyte content and activity expressed in terms of mg of mononuclear leukocyte protein.

number of normals assayed was small, results of the assay in mixed leukocytes were entirely consistent with the findings from lymphocytes (Fig. 1). That the reduced pyruvate carboxylase activities observed are genuine and not due to a transient phenomenon peculiar to one particular blood sample is demonstrated convincingly by the observation that the patient's brother was normal and the mother and sister carriers, in two different blood samples drawn more than 8 months apart and with different white cell isolation procedures.

The demonstration of reduced pyruvate carboxylase activity (34, 42, 67, and 40% of the lowest normal) in the fibroblasts of obligate carriers from two separate families with hepatic pyruvate carboxylase deficiency indicates that the observation of reduced pyruvate carboxylase activity in that tissue may be a common finding in this disease and not just peculiar to one case. However, more obligate carriers must be examined before any conclusions can be drawn about the universality of this observation.

The mitochondrial PEPCK activity in leukocytes from the patient with PC^{Portland} deficiency is somewhat reduced, and is significantly reduced in the patient's fibroblasts (Table 2). That this reduction is not common to all patients with pyruvate carboxylase deficiency, however, is shown by the normal or near normal PEPCK in the fibroblasts from the patient with PC^{St. Louis} deficiency. As was discussed (4), all the parameters for the proper assay of mammalian PEPCK may not be known and this may offer an explanation for the variation in observed values.

A comparison of the pyruvate carboxylase activities presented here with those of DeVivo *et al.* (6) for the fibroblasts from the family with PC^{St. Louis} deficiency is worthy of comment. The present study confirms their finding of a deficiency in the patient and reduced activity in the parents, but the absolute values for patient, parents, and normals is different in the two studies. This difference in activity from laboratory to laboratory demonstrates the absolute necessity of establishing normal enzyme values in the same laboratory where patients or potential carriers are to be examined. It also underscores the value of the Utter and Keech (13) Ballard and Hanson (5) assay of pyruvate carboxylase activity because without the extreme sensitivity provided by that method, the detection of carriers of pyruvate carboxylase deficiency in lymphocytes would be much more difficult, if possible at all.

The very low activity of lymphocyte pyruvate carboxylase requires rigorous attention to the details of the assay in order to determine carriers. However, with the use of this tissue, diagnosis is possible in 2-3 days and the individual is exposed to nothing more invasive than venipuncture. In contrast, the assay in fibroblasts requires the more invasive procedure of skin biopsy and several weeks to acquire fibroblasts. However, because fibroblasts contain about 10 times as much pyruvate carboxylase as do lymphocytes, and because as many fibroblasts can be grown as is required, the enzyme is easier to assay in that tissue. Fibroblasts

have the further advantage of being several generations removed from the pathologic environment, in the case of a patient, and thus, one can be more certain that a reduced enzyme level is due to a genetic defect and not secondary to some other pathologic process as might be the case with circulating peripheral lymphocytes. Although it is possible to assay the lymphocyte pyruvate carboxylase in preparations of mixed leukocytes, the author prefers the use of the separated lymphocyte plus monocyte fraction. If pyruvate carboxylase deficiency is suspected in a patient, it would be wise to obtain both a skin biopsy, and a lymphocyte plus monocyte fraction, if possible.

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