Pyruvate Carboxylase Deficiency: A Benign Variant with Normal Development

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ABSTRACT. The devastating nature of a pyruvate carboxylase deficiency is underscored by the uniformly fatal outcome of the neonatal (French) type and the severely disabling and ultimately fatal outcome of the infantile (North American) type. We report a 7-y-old girl with metabolic and biochemical features of the North American phenotype. Remarkably, the clinical course has been benign with preservations of motor and mental abilities. The residual enzyme activity in cultured skin fibroblast homogenates was 1.8% and cross-reacting material was present in normal abundance and electrophoretic mobility. She has had several episodes of metabolic acidosis with elevated lactate, pyruvate, alanine, β -hydroxybutyrate, acetoacetate, lysine, and proline values, and undetectably low aspartate concentrations. These crises have been managed by rehydration and bicarbonate therapy. We are unable to provide a satisfactory explanation for the uniquely benign clinical course that has been experienced by this patient. (*Pediatr Res* 30: 1–4, 1991)

Abbreviations

CRM, cross-reacting material
MW, molecular weight
PC, pyruvate carboxylase
SSC, standard saline citrate
TBS, Tris-buffered saline

PC is a mitochondrial matrix enzyme that catalyzes the ATP-dependent fixation of CO_2 to pyruvate yielding oxaloacetate (1–3). This reaction is essential to the function of the Krebs cycle, gluconeogenesis (4), and lipogenesis (5, 6). The enzyme is expressed in brain, liver, kidney, adrenal gland, lactating mammary gland, fibroblasts, white blood cells, adipose, and other tissues (5, 7–9). Low activities are found in skeletal muscle. Tissue-specific isoenzymes are not known. The PC holoenzyme is a tetramer composed of four identical monomers (α_4) each with a MW of 125 kD (10). Partial cDNA clones corresponding to the 3' terminal of PC mRNA are available (6, 11), and the gene is located on the long arm of chromosome 11 (6).

Thirty-four patients with PC deficiency have been reported. Sixteen were severely ill in the neonatal period (French phenotype) with lactic acidosis, citrullinemia, hyperlysinemia, and

hyperammonemia (12–22). Essentially all these patients died within the first 4 mo of life. Eighteen patients had clinical symptoms with lactic acidosis during infancy (North American phenotype) (7–9, 18, 23–31). These patients had severe mental and motor developmental delay and death during infancy or early childhood.

We have encountered a patient, now 6½ years of age, with severe PC deficiency documented in cultured skin fibroblasts who has had normal mental and motor development. To our knowledge, this child is the only patient with a “benign form” of PC deficiency.

CASE REPORT

This 7-y-old girl is the only child of a nonconsanguineous marriage. The full-term pregnancy, labor, and delivery were uncomplicated. Birth weight was 4500 g. Growth and development during the first year of life were normal. At 7 mo of age, she was hospitalized briefly for vomiting and dehydration, and was hospitalized again at 14 mo for evaluation of a 1-d history of vomiting and rapid respirations. Physical examination at that time revealed an acutely ill infant. Body weight and length were above the 95th percentile, and the head circumference was 48 cm (90–95th percentile). Although afebrile, her respiratory rate was 60/min. Auscultatory and radiographic findings were consistent with bronchiolitis. Serum glucose was 14 mM, sodium 132 mM, chloride 111 mM, bicarbonate 1.5 mM, and urea nitrogen 4.1 mM. Blood gases were 1.7 kPa (13 mm Hg) PCO_2 and 17.3 kPa (130 mm Hg) PO_2 . The pH was 6.89 and the base excess -28.5 mM. Urinary pH was 5.0; large quantities of ketones were present. Treatment included oxygen, bronchodilators, sodium bicarbonate, and a single dose of intramuscular insulin. She responded quickly to treatment and was discharged on the 4th day of hospitalization. One wk later she was readmitted for a metabolic evaluation. Serum chemistry profile was normal, but the blood lactic acid value was 5.5 mM (normal 0.5 to 2.2). Blood ammonia was normal. Plasma amino acid profile by anion exchange chromatography, however, revealed elevated alanine [852 μM (normal 99 to 313)], lysine [160 μM (normal 45 to 144)], and proline [281 μM (normal 51 to 185)]. Citrulline was normal and aspartate was undetectably low. The 24-h urine lactic acid content was 1.48 mg/mg creatinine (normal <0.1) as analyzed by gas chromatography/mass spectrometry.

The child was discharged but readmitted 7 d later with a 24-h history of decreased appetite, restlessness, and unsteady gait. On admission she was afebrile, tachypneic (60/min), tachycardic (178/min), and ataxic. Serum glucose was 8 mM, bicarbonate 4.9 mM, lactic acid 7.3 mM, and pyruvic acid 0.306 mM (normal <0.15). She responded rapidly to i.v. fluids and bicarbonate, and was discharged after 2 d of hospitalization.

The child was hospitalized again at 16, 25, 31, and 47 mo of age for acute episodes of lactic acidosis. Each episode was preceded by a brief illness and decreased appetite and responded

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rapidly to i.v. fluids and bicarbonate therapy. Several milder episodes were treated at home with oral bicarbonate therapy and increased fluids. At 47 mo of age, the child weighed 24.5 kg (>95th percentile) and her neurologic development was normal except for a mild dysarthria. When last seen at 6½ y of age, the child was attending the first grade and performing all age-appropriate activities. She was doing well in school except for some difficulty with mathematics. She continued to be free of severe episodes of lactic acidosis, but was given supplemental oral fluids and bicarbonate whenever she was febrile. She was a large child whose height was 132 cm (>95th percentile) and who weighed 42 kg (>95th percentile). Except for macrosomia, obesity, and mild dysarthria, her physical and neurologic examinations were normal.

MATERIALS AND METHODS

Tissue culture supplies were purchased from Gibco Laboratories (Grand Island, NY). Laboratory reagents and chemicals were purchased from Sigma Chemical Company (St. Louis, MO), SDS-polyacrylamide electrophoresis reagents and the streptavidin-horseradish peroxidase conjugate from Bio-Rad (Rockville Center, NY), DNA polymerase and NADH from Boehringer-Mannheim Biochemicals (Indianapolis, IN), and ³²P-nucleotides from New England Nuclear (Wilmington, DE).

Fibroblasts were cultured from biopsied skin specimens and trypsinized as described previously (32). The cell pellet was resuspended in nine volumes of homogenizing buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 2 mM EDTA). The suspension was homogenized in a glass tube with a Teflon pestle for 10 passes at 300 rpm. Triton X-100 was added to a 0.01% homogenate, which was allowed to sit on ice for 30 min before assay.

Spectrophotometric assays were used to measure succinate cytochrome c reductase, NADH cytochrome c reductase, NADH dehydrogenase, succinate dehydrogenase, citrate synthase, and cytochrome c oxidase as described previously (33). Reduced cytochrome c was freshly prepared before each experiment by adding a few grains of sodium hydrosulfite (dithionite) to a 1% cytochrome c solution in 10 mM K-phosphate buffer, pH 7.0.

The activities of PC and the pyruvate dehydrogenase complex were determined after scraping from the culture plate at the time of assay as described previously (9, 32).

Isolation of mitochondria, electrophoresis of mitochondrial proteins, and transfer of proteins to nitrocellulose membrane were performed as described previously (32), except that a 7% SDS-polyacrylamide gel was used. The membrane was immersed in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) for 10 min, 3 g/100 mL Carnation skim milk powder-0.5 g/100 mL gelatin in TBS for 1 h, 0.05 vol% Tween-20 in TBS twice for 5 min, and subsequently incubated in the presence of a streptavidin-horseradish peroxidase conjugate (1/250 dilution in 1 g/100 mL gelatin in TBS) for 2 h. The membrane was washed again twice in Tween-20 in TBS for 5 min and in TBS also for 5 min. Color was developed by adding 0.1 g/100 mL diaminobenzidine, 0.1 g/100 mL imidazole, and 0.015% H₂O₂ in TBS.

Nuclear pellets obtained from 700-g fibroblast homogenates were used for isolation of DNA. The pellets were resuspended in 5 mL of 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 200 µg/mL SDS, and 200 µg/mL proteinase K and placed on a revolving wheel at 37°C for 12 h. After phenol extraction, 1/10 vol 3 M Na acetate (pH 5.1) and two vol ethanol were added, and DNA was spooled out and resuspended in 1 mL of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

Genomic DNA (10 µg) was digested with 3 U/µg of restriction endonucleases (*Hind*III, *Eco*RI, *Bgl*II, and *Bam*HI) at 37°C for 4 h. The DNA was subjected to electrophoresis in 1% agarose and transferred to nitrocellulose (34). Before transfer, the gel was denatured with 0.25 M HCl for 15 min and 0.5 M NaOH/1.5 M NaCl for 30 min and neutralized with 0.5 M Tris-HCl/1.5

NaCl (pH 7.0–7.5) for 30 min. Transfer proceeded for 20 h. The filter was heated at 80°C for 2 h *in vacuo*. Prehybridization was carried out in a solution containing 40% formamide, 0.6 M sodium chloride and 0.06 M sodium citrate (4 × SSC), 5 × Denhardt's solution, 0.5 mg/mL denatured salmon sperm DNA, 0.2% SDS, and 0.1 M Tris-HCl (pH 7.4) at 42°C for 6 h. The filter was then hybridized overnight at 42°C in 40% formamide, 4 × SSC, 1 × Denhardt's, 0.1 M Tris-HCl (pH 7.4), 2.5 g/100 mL dextran sulfate, and 10⁶ cpm/lane of ³²P-labeled PC probe prepared from denatured double-stranded cDNA using the random priming method. The filter was washed in 4 × SSC, 0.1% SDS, then in 2 × SSC, 0.1% SDS, and in 0.2 × SSC, 0.1% SDS (at room temperature for 15 min). The filter was blot-dried and hybridization signals were visualized by autoradiography using a Dupont Cronex Lightning Intensifying screen. Protein was determined by the Lowry method (35). A cDNA probe, 1.1 kbp in length, corresponding to the 3' terminal of PC mRNA was a generous gift of Svend Freytag (University of Michigan Medical School, Ann Arbor, MI) (6).

RESULTS

Mitochondrial enzyme activities measured in cultured skin fibroblast homogenates of our patient and controls are shown in Table 1. PC residual activity in the patient's cells was only 1.8% of the control cell activity. The activities of other mitochondrial enzymes were normal.

The electrophoretic profile of biotin-containing mitochondrial proteins is shown in Figure 1. Three discernible bands were visualized in the control cells and PC-deficient cell lines. The band with the highest MW represents the biotin-containing subunit of PC (MW = 125 kD); the doublet corresponds to the α-subunits of β-methyl-crotonyl CoA carboxylase (MW = 75 kD) and propionyl CoA carboxylase (MW = 73 kD). All cell lines, including our patient's, showed the same pattern. There were no differences in abundance or mobility of the PC subunits between different cell lines.

The cleavage pattern of the digested genomic DNA extracted from fibroblasts of our patient, three other PC-deficient patients with the North American phenotype, and control samples were identical (data not shown).

DISCUSSION

Two types of PC deficiency have been described in the literature. The French phenotype presents in the neonatal period and is characterized clinically by anorexia, weak cry, convulsions, stupor, hyporeactivity, hypotonia, abnormal respiratory pattern, subsequent hypertonia, extrapyramidal tract signs, and severely retarded mental and motor development. Biochemical charac-

Table 1. Cultured skin fibroblast enzyme activities*

Enzyme	Patient†	Controls (mean ± SD)‡
Pyruvate carboxylase	0.03	1.62 ± 0.39
Pyruvate dehydrogenase complex	1.86	1.72 ± 0.35
Cytochrome c oxidase	37.7	50.3 ± 11.6
NADH dehydrogenase	633	910 ± 169
NADH cytochrome c reductase (total)	190	210 ± 30
Succinate cytochrome c reductase	11.6	16.7 ± 4.6
Succinate dehydrogenase	9.43	12.3 ± 3.2
Citrate synthase	32.8	76.3 ± 15.1

* nm·min⁻¹·mg protein⁻¹ activities represent: for pyruvate carboxylase, nmol HCO₃ fixed; for pyruvate dehydrogenase, complex nmol CO₂ produced; for citrate synthase, nmol CoASH produced; and for other enzymes, nmol substrate oxidized.

† Enzyme activity represents the average value of two assays.

‡ Pyruvate carboxylase: n = 113; pyruvate dehydrogenase: n = 112; other assays: n = 41.

Fig. 1. Mitochondria were isolated from cultured skin fibroblasts and electrophoresed on SDS-7% polyacrylamide gel. The proteins were transferred to nitrocellulose membrane and labeled with streptavidin-peroxidase (see Materials and Methods for detail). MW markers (kD) are shown in the left margin. Lanes 1 and 7 are control samples; lane 2 is the patient with the benign PC variant; and lanes 3–6 are patients with the classic forms of fatal PC deficiency. Normal abundance of CRM for PC and for the α -subunits of β -methylcrotonyl CoA carboxylase (MCC) and propionyl CoA carboxylase (PCC) is present in all seven lanes.

teristics include lactic acidosis, increased lactate/pyruvate ratio, citrullinemia, hyperlysinemia, hyperammonemia, and ketosis. Most of these patients died within the first 4 mo of life. Patients with the North American type become symptomatic between 2 and 5 mo of age, associated with delayed mental and motor development, failure to thrive, apathy, hypotonia, pyramidal tract signs, ataxia, nystagmus, and convulsions. Episodes of acute vomiting, tachypnea, and acidosis characteristically are precipitated by metabolic or infectious stress. Serum lactate and pyruvate values usually are less increased in the North American type.

Patients of both types suffer from severe mental and motor retardation and most die in infancy. Robinson *et al.* (18) described two cases, initially symptomatic at 2 and 5 mo, respectively, who survived until 5 y of age. One of the patients described by Tada *et al.* (29) was 7½ y old when he died. Our patient was normal until 7 mo of age, but subsequently developed several acute episodes of anorexia, vomiting, tachypnea, tachycardia, and ataxia. She had metabolic acidosis and increased serum lactate, pyruvate, β -hydroxybutyrate, acetoacetate, alanine, lysine, and proline; the aspartate value was undetectably low. She was PC-deficient as shown by the decreased catalytic activity in her cultured skin fibroblasts (Table 1). The metabolic profile is similar to previously reported PC-deficient patients with the North American phenotype, but clinically she has continued to show normal motor and mental development.

More than half of the patients with the French phenotype had absence of PC protein and the corresponding mRNA. Patients with the North American phenotype generally have CRM (CRM-positive) (11, 18). Our patient is CRM-positive (Fig. 1), as expected by the onset of symptoms in early infancy.

PC serves an important role in cell metabolism. First, it serves an anaplerotic function replenishing the Krebs cycle intermediates and, second, it serves a role in gluconeogenesis. In general, it is believed that the lost anaplerotic function is more relevant to the metabolic crises experienced by these patients. This impression is evidenced by the low aspartic acid concentrations in these patients, inasmuch as this amino acid is in equilibrium with oxaloacetate. Aspartic acid insufficiency is particularly severe in the French phenotype and is responsible for the citrullinemia and hyperammonemia. Aspartic acid supplementation may mitigate some of these systemic metabolic disturbances (36).

The rather benign course of PC deficiency in our patient remains unexplained. Tissue heterogeneity would provide a convenient explanation for this patient's condition. However, Atkin *et al.* (7, 8) measured PC activity in liver, kidney, brain, cultured skin fibroblasts, and white blood cells of one patient and found it to be deficient in all tissues, suggesting that only one molecular form of PC exists in human tissues. Animal studies also confirm

this observation (10). These observations are compatible with the known fact that PC is a tetramer containing four identical subunits. To invoke tissue heterogeneity as the mechanism accounting for the benign clinical state, we would need to propose that PC exists in different isoforms in the affected and unaffected tissues. Differential splicing of mRNA transcripts in these diverse tissues would represent one possible explanation.

Synthesis of an alternative metabolic intermediate, particularly in the brain, to replenish the Krebs cycle intermediates might explain the benign course. One possibility is that the brain enzymes constituting the "glutamine loop" could produce more α -ketoglutarate from glutamine to compensate for the decreased oxaloacetate formation (27). This metabolic adaptation probably happens in PC-deficient patients, as witnessed by the decreased glutamine in autopsied brain of one patient and in cerebrospinal fluid and plasma of four living patients with PC deficiency (27). Why this metabolic adaptation would be more efficient in our patient and less effective in the other reported cases cannot be answered.

It also remains theoretically possible that a fetal PC isoform persists during early infancy and protects the developing organs, including the brain. However, we have no evidence to support this hypothesis. In fact, Robinson *et al.* (17) have shown evidence for brain involvement during fetal development in a PC-deficient patient.

The enzyme activity measured *in vitro* may not be a true reflection of the enzyme activity *in vivo*. However, the intermittent metabolic crises and the blood and urine metabolite profiles are entirely compatible with PC deficiency. Of interest in this regard is the fact that the patient never suffered any hypoglycemic episodes. In fact, when she presented at 14 mo, the blood glucose concentration was elevated and she was treated with one dose of insulin. This metabolic disturbance is distinctly unusual in patients with PC deficiency. Although we have no explanation for the patient's hyperglycemia during the metabolic crises, it is again intriguing to speculate about the possible existence of tissue-specific isoforms, but available data militate against this speculation.

At this point we can only report that our patient has a CRM-positive PC deficiency, but, for reasons that remain obscure, her clinical course has been rather benign and her psychomotor functioning at 7 y of age is essentially normal.

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