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B H Robinson, ... , T Coskun, P W Stacpoole

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## Research Article

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# Defects in the E<sub>2</sub> Lipoyl Transacetylase and the X-lipoyl Containing Component of the Pyruvate Dehydrogenase Complex in Patients with Lactic Acidemia

Brian H. Robinson,\*\* Nevena MacKay,\* Roumyana Petrova-Benedict,\* Imran Ozalp,<sup>§</sup> Turgay Coskun,<sup>§</sup> and Peter W. Stacpoole<sup>||</sup>

\*Research Institute, The Hospital for Sick Children, and †the Departments of Pediatrics and Biochemistry, University of Toronto, Toronto, Ontario; §Department of Pediatrics, Hacettepe University, Ankara, Turkey; ||Departments of Medicine and Pharmacology, Division of Endocrinology and Metabolism, University of Florida, Gainesville, Florida 32610

## Abstract

Three patients with chronic lacticacidemia and deficiency of the pyruvate dehydrogenase complex demonstrated in cultured skin fibroblasts showed abnormalities on Western blotting with anti-pyruvate dehydrogenase complex antiserum which were not located in the E<sub>1</sub> ( $\alpha$  and  $\beta$ ) component of the complex. One of these patients had an enzymatically demonstrable deficiency in the E<sub>2</sub> dihydrolipoyl transacetylase segment of the complex and very low observable E<sub>2</sub> protein component on Western blotting of fibroblast proteins. The other two patients had abnormalities observable in the X component but no observable reduction in either E<sub>1</sub>, E<sub>2</sub>, or E<sub>3</sub> enzymatic activities. One patient appeared to have a missing X component while the other had two distinct bands where X should be on Western blotting of fibroblast proteins. All three patients appeared to have severe clinical sequelae resulting from these defects. This is the first time that defects in either the E<sub>2</sub> or the X component of the pyruvate dehydrogenase complex have been observed in the human population. (*J. Clin. Invest.* 1990. 85:1821–1824.)  
lactic acidemia • pyruvate dehydrogenase deficiency

## Introduction

The mammalian pyruvate dehydrogenase complex (PDHC)<sup>1</sup> differs from the complex in *Escherichia coli* and from the other mammalian  $\alpha$ -keto-acid hydrogenases by the presence of a 53-kD protein that has been labeled protein X (1, 2). This protein, whose mobility on sodium dodecyl sulfate polyacrylamide gels is slightly greater than the E<sub>3</sub> component lipoamide dehydrogenase ( $M_r = 53,000$ ), resembles the E<sub>2</sub> dihydrolipoyl transacetylase protein in that it bears a lipoyl group attached to a lysine residue (3, 4). This lipoyl residue is capable of being acetylated when the complex is treated with pyruvate (1, 2), though protein X itself has no innate dihydrolipoyl transacetylase activity. The study of human pyruvate dehydrogenase complex deficiency has shown that defects in the E<sub>1</sub> compo-

nent are most common, with a smaller number of E<sub>3</sub> defects (5). No case with defective E<sub>2</sub> transacetylase activity has ever been described.

In the course of examining skin fibroblast cultures from patients with chronic lacticacidemia we have accumulated a group of cell lines from some 60 patients with either severe or partial deficiency of the pyruvate dehydrogenase complex (activities 0.5–60% of controls). We have reported recently in a review that in 51 patients we have the data to define the subcomponent of the complex responsible for the deficient activity by enzymatic analysis of the three catalytic components (6). Of these, 46 were deficient in the E<sub>1</sub> component, 1 in the E<sub>2</sub> component (patient T.B. below) and 4 in the E<sub>3</sub> component (6). We have carried out Western blotting analysis on the 51 patient cell lines and on a few patient cell lines whose results did not give a clear indication of the defective component. This latter group produced two patient fibroblast cell lines that gave an unusual pattern on Western blotting with anti-PDHC antibody that seemed to involve protein X. We report here the clinical presentation, the enzymatic activities and the immunoreactive protein profile of the PDH complex in these three patients with putative defects in the E<sub>2</sub> or X components.

## Methods

### Case reports

**Patient 1.** T.B. is a girl who presented at 2 wk of age with hyperammonemia and profound lactic acidosis. She was the product of a 36–37 wk pregnancy of a 17-yr-old black mother. 10 d postpartum she developed what appeared to be respiratory distress. Urine organic acids, plasma uric acid, and plasma amino acids were uninformative. She was treated at the University of Florida, Neonatal Intensive Care Unit with dichloroacetate (50 mg/kg doses). Initial administration caused a dramatic decrease in blood lactate, though doses administered later were not as efficacious.

On carbohydrate restriction and bicarbonate supplementation (bicitra) she has stabilized metabolically with blood lactates at 3–5 mM. However, at 3½ yr of age she is now profoundly psychomotor retarded (functions at 1–2-mo age level) and is microcephalic. She is maintained on a high fat low carbohydrate diet with a typical lactate of 3–5 mM.

**Patient 2.** S.Y. was first seen at the age of 5 mo because of poor development. He was found to be hypotonic and lacked head control. By the age of 18 mo hypotonia was still marked and he could not sit unsupported. His parents are first cousins. There was a 5-yr-old sibling who was mentally retarded being 3rd percentile for head circumference and weight. Blood ammonia (76  $\mu$ M) was slightly increased, as was alanine. Blood lactate varied between 5.9 and 9.1 mM and blood pyruvate was also increased. Treatment with thiamine (20 mg daily) did not bring about any improvement. At 4 yr of age he was severely mentally and motor retarded. He could neither walk nor speak.

**Patient 3.** K.S. was born to nonconsanguineous Japanese parents. This boy did not have any problems until 3 yr of age when he was noted to have an unsteady gait and difficulty with running and frequent falling. Intellectually and linguistically his development has been

Address reprint requests to Dr. Robinson, Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.

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1. Abbreviations used in this paper: PDHC, pyruvate dehydrogenase complex.

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normal; his height, weight, and head circumference have all been appropriate for age. Poor fine motor and gross motor coordination was evident at 5½ yr of age. Blood lactate (2.5–9 mM) and pyruvate were above the normal range of values on numerous occasions. At the age of 5½ yr computed tomography (CT) scanning revealed bilateral basal ganglia lucencies and symmetrical lacunae in the area of the putamen. Blood alanine was elevated and urine organic acid analysis repeatedly showed the presence of leucine metabolites, 3-hydroxyisovaleric, 3-methylglutaconic, and 3-hydroxy-3-methylglutaric acids as well as moderate amounts of citric and  $\alpha$ -ketoglutaric acid. Thiamine (25 mg daily) administration appeared to improve levels of blood lactate while having no effect on clinical status.

### Materials

**Assay of enzyme activities.** Pyruvate dehydrogenase complex activity was measured in cultured skin fibroblasts before and after activation with 5 mM dichloroacetate by the method of Sheu, Hu, and Utter (7). The subcomponents of the complex were measured as follows: E<sub>1</sub> by the method of Robinson, Taylor, and Sherwood (8), E<sub>2</sub> by the method of Butterworth et al. (9) and E<sub>3</sub> by the method of Reed and Wilms (10) modified as described in (8).

**Skin fibroblast cultures.** Skin fibroblast cultures from patients with lacticacidemia were grown in  $\alpha$ -MEM medium supplemented with 10% fetal calf serum. Cell lines with deficient pyruvate dehydrogenase complex activity were identified and assayed further for the components of the complex.

**Western blotting.** Western blotting was performed essentially by the procedure of Brada and Roth (11) except that peroxidase labeled final antibody was used for color development. Antibodies used were those raised in rabbits against bovine heart pyruvate dehydrogenase complex or pig heart lipoamide dehydrogenase (Sigma Chemical Co.).

Each lane for blotting was derived from two plates of confluent fibroblasts ( $2.6 \times 10^6$  cells) as follows. After removal of the culture medium the plates were extracted for 5 min with 1 ml 0.1% digitonin, 0.25 M sucrose, 1 mM EDTA, 20 mM MOPS (morpholinopropane sulphonate), pH 7.4. This digitonin extract was discarded and the cells were then extracted with 1% Triton X-100, 1 mM PMSF, 30 mM Hepes, 0.15 M NaCl, pH 7.4. This extract was subjected to acetone precipitation and after centrifugation the resulting protein pellet was solubilized by boiling in 1% SDS, 0.2 M DTT, 10% glycerol, 30 mM Tris HCl, pH 6.8, and loaded on to a 10% polyacrylamide slab gel. Transfer of protein was accomplished by electroblotting (Bio-Rad Laboratories, Richmond, CA) onto nitrocellulose support membranes.

These were blocked with 3% gelatin-TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.5) for 1 h, followed by an overnight incubation with primary antibody (1:1,000). Incubation in secondary antibody

(1:3,000) was for 3 h using goat anti-rabbit IgG horseradish peroxidase (Bio-Rad Laboratories).

### Results

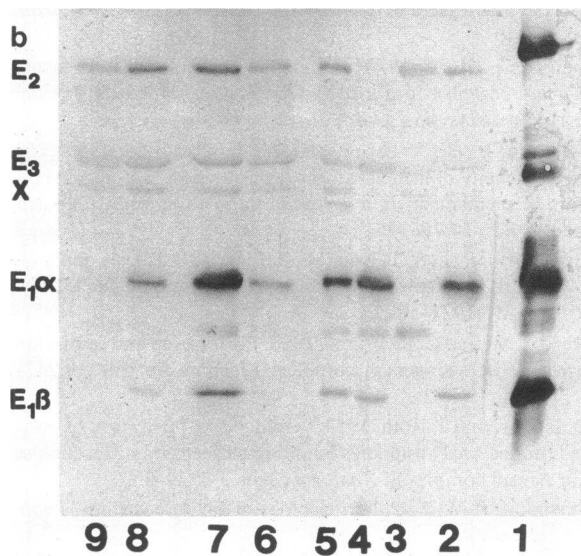
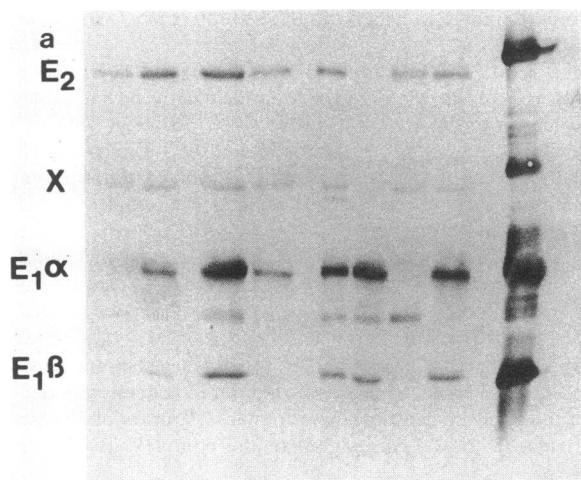
The three patients were found to have deficiency of the pyruvate dehydrogenase complex as judged by the activity in cultured skin fibroblasts (Table I). Patients 1 and 2 had 23.7 and 12.3%, respectively, of the activities found in controls when assayed for PDH complex activity after activation by dichloroacetate. Patient 3, however, had 55% of control activity. Measurement of the activity was performed on two separately thawed fibroblast cultures and gave similar values. An independent assay by Dr. R. Butterworth (St. Luc Hospital, Montreal) gave an almost identical result (56% of control activity). For comparison three cases of PDH complex deficiency in which the E<sub>1</sub> component is deficient are included in Table I. Measurement of the subcomponents of the complex, the E<sub>1</sub> decarboxylase, the E<sub>2</sub> transacetylase and the E<sub>3</sub> lipoamide dehydrogenase, showed that patients 2 and 3 had completely normal activity of all three components. Patient 1, however, had an E<sub>2</sub> activity that was 32% of the control value. Thus the subcomponents of the PDH complex all appeared to be normal in patients 2 and 3, despite the decreased activity of the complex.

Western blotting of the proteins present in cultured skin fibroblasts was carried out using an antibody generated to pig heart pyruvate dehydrogenase complex. This antibody recognizes the components E<sub>1</sub> $\alpha$ , E<sub>1</sub> $\beta$ , E<sub>2</sub>, and X (Fig. 1 a). Redeveloping with an antibody against pig heart lipoamide dehydrogenase revealed the E<sub>3</sub> component (Fig. 1 b). The pattern for patient 1 T.B. (lane 4) showed a discrete absence of the E<sub>2</sub> transacetylase component with E<sub>1</sub> $\alpha$ , E<sub>1</sub> $\beta$ , and E<sub>3</sub> as normal. The amount of X was also reduced in this patient. Patient 3, K.S. (Fig. 1, a and b, lane 5), had normal bands for E<sub>2</sub>, E<sub>1</sub> $\alpha$ , and E<sub>1</sub> $\beta$ , but displayed a double band for the X-component, one of normal mobility and one of increased mobility, suggesting decreased molecular mass. Whether this additional band was due to proteolytic cleavage of an abnormal X protein, due to the de novo synthesis of a truncated version of the X-protein, or due to a change in charge or conformation affecting mobility could not be elucidated. Patient 2 (S.Y., lane

Table I. Activities of the Pyruvate Dehydrogenase Complex Components in Patients and Controls

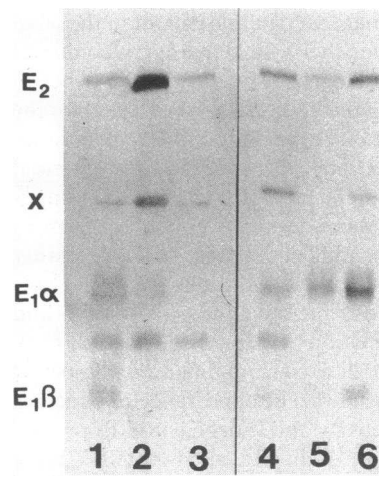
Index cases	Pyruvate dehydrogenase complex				
	Native	+DCA	E <sub>1</sub>	E <sub>2</sub>	E <sub>3</sub>
	nmol/min per mg		nmol/h per mg	nmol/min per mg	
Patient 1, T.B.	0.318±0.054 (6)	0.322±0.053 (6)	1.54±0.29 (5)	3.69±1.21 (5)	15.1±3.0 (3)
Patient 2, S.Y.	0.191±0.027 (7)	0.167±0.030 (7)	1.39±0.26 (3)	14.4±2.2 (4)	11.5±1.2 (2)
Patient 3, K.S.	0.677±0.166 (6)	0.753±0.094 (7)	1.11±0.30 (2)	13.3±4.5 (5)	10.6±1.3 (2)
E <sub>1</sub> -deficient patients					
R.S. (2056)	0.354±0.037 (8)	0.368±0.038 (8)	0.49±0.11 (5)	8.71±0.96 (2)	7.9±2.7 (2)
O.P. (2583)	0.092±0.022 (5)	0.068±0.037 (5)	0.21±0.06 (3)	8.48 (1)	13.5±2.0 (2)
J.L. (2641)	0.050±0.031 (5)	0.044±0.027 (5)	0.26±0.07 (3)	6.85±3.1 (3)	9.8±1.3 (3)
Controls					
(1206)	0.932±0.076 (6)	1.368±0.085 (6)	1.33±0.21 (3)	10.9±1.65 (4)	9.45±0.73 (3)
(2176)	1.071±0.067 (11)	1.360±0.065 (11)	Not determined	11.73±2.1 (3)	13.1±2.2 (3)

Values are given as the mean of a number of determinations (given in parentheses) ± the standard error of the mean.



**Figure 1.** Western blotting of PDHC-deficient cultured skin fibroblast extracts with anti-PDH complex antibody. Each cell line was subjected to digitonin and Triton X-100 extraction and the Triton extracts run out on a 10% polyacrylamide gel. This gel was electroblotted onto Hybond support matrix and blotted with anti-PDH complex antibody. Immunoreactive proteins were visualized by goat anti-rabbit IgG horseradish peroxidase staining. (a) Anti-PDHC alone. (b) The same blot was redeveloped with antilipoamide dehydrogenase (E<sub>3</sub>) antibody. (Lane 1) PDHC protein (bovine heart 5 μg). (Lane 2) Control cell line 1286. (Lanes 3, 6-9) PDH-E<sub>1</sub> deficient cell lines 1861, 2052, 2436, 2572, 2653. (Lane 4) Patient 1, T.B. (Lane 5) Patient 3, K.S.

5) appeared to have a missing X component (Fig. 2) while E<sub>2</sub>, E<sub>1</sub>α, and E<sub>1</sub>β appeared to be reduced to below normal in this patient. In order to compare the immunoblotting signals for this group of patients with other PDHC deficient patient cell lines, a cohort of PDH-E<sub>1</sub> deficient cell line was included in these experiments. Lanes 3, 6-9 for Fig. 1, a and b were fibroblast proteins from patients with PDH-E<sub>1</sub> deficiency, as were lanes 1-4 of Fig. 2. Lanes 2 and 6 for Figs. 1 and 2, respectively, were control cell lines. Many of the deficient cell lines had reduced amounts of E<sub>1</sub>α and E<sub>1</sub>β protein (6). This shows that true defects in the E<sub>1</sub> component (α or β) while affecting



**Figure 2.** Western blotting of PDHC deficient cultured skin fibroblasts. Experiments were carried out as described for Fig. 1 using anti-PDHC antibody. Lanes 1-4, PDH-E<sub>1</sub> deficient cell lines 2645, 2641, 2583, 2360. Lane 5, Patient 2, S.Y. Lane 6, control cell line 1286.

the stability of the protein subunits of E<sub>1</sub>, do not cause any abnormalities in either E<sub>2</sub> or X even when there is almost no E<sub>1</sub> present as in lanes 2 and 9 (Fig. 1).

### Discussion

We have demonstrated here the first documented case of E<sub>2</sub>-dihydrolipoyl transacetylase deficiency in a case of lacticacidemia with reduced activity of the pyruvate dehydrogenase complex. Western blotting, showing decreased intensity of bands for the E<sub>2</sub> and X proteins of the complex in the patient, is strong corroborative evidence for the presence of a transacetylation abnormality. In addition we have for the first time demonstrated that two patients with PDH complex deficiency have a primary abnormality in the X component. The evidence for this is based on two observations, firstly on the normal subcomponent activities of E<sub>1</sub>, E<sub>2</sub>, and E<sub>3</sub> within the complex, and secondly on the abnormalities seen with the X component on Western blotting. Since these are totally new findings they will be discussed in the context of clinical presentation and in the context of the role of the X component in the activity of the PDH complex.

Patients 1 and 2 were children who suffered severely from psychomotor retardation and moderate lacticacidemia, patient 2 having a lower PDH complex activity than patient 1. In the case of patient 2 the suspicion of a familial inherited defect was high; the parents were first cousins and one elder sibling was mentally retarded. In all respects other than biochemical analysis these children could not be distinguished on clinical grounds from those with a defect in the PDH-E<sub>1</sub> decarboxylase component (5) many of whom suffer from moderate lacticacidemia and profound psychomotor retardation. Many of these patients, especially those with good residual activity of the PDH complex also show a clinical response to dichloroacetate administration (12). Patient 3 resembled the cases of PDH deficiency in whom there is a mild course and in whom the prominent clinical features are ataxia and carbohydrate sensitive lacticacidemia (5, 6). The fact that the residual activity was high (55%) in fibroblasts coupled with the knowledge that a defective component is present in the PDH complex indicates that even partial defects in PDH can be harmful. This group with ataxia only consists of all boys and have been shown to be linked to defects in the E<sub>1</sub>α component of the PDH-complex which is encoded by the X-chromosome (6). However, the

central nervous system damage seen in this patient in the basal ganglia would be atypical for this X-linked group who do not develop lesions in the central nervous system. The presence of one normal and one low relative molecular weight band for the X component suggests that one explanation for the high residual activity in the complex may be attributed to the normal appearing X. Some time ago the possibility that the X component of the PDH complex was a degradation product from E<sub>2</sub> breakdown was shown to be unlikely because of the dissimilar appearance of tryptic digests of the two proteins (1, 2). However, the similarity in the construction and behavior of E<sub>2</sub> and X still suggest a strong association between them. Matuda et al. have suggested that they are derived from differentially spliced transcripts of the same gene (13). The appearance of patient 1 who lacks transacetylase activity and is deficient in E<sub>2</sub> protein suggests some linkage because of the decreased protein X band seen by immunoblotting in this patient. On the other hand, patient 2 has a missing band for protein X but a reasonable band for the E<sub>2</sub> protein. This could fit with the "one gene—two proteins" theory if the mutations had occurred at critical splice sites, but it would also be quite compatible with separate genes encoding the X and E<sub>2</sub> proteins. Interestingly, the abnormality present in E<sub>2</sub> affects measurable transacetylase activity but those in protein X do not. This suggests that X itself does not contribute significantly to the cellular dihydrolipoyl transacetylase activity despite the fact that the lipoyl groups on X can be acetylated (1, 2). If the PDH complex could be purified from patient derived cell lines in sufficient quantity to allow detailed study more information could be gained about the role of the two lipoyl proteins in the complex. The cloning and sequencing of X itself should be able to clarify much of the relationship between X and E<sub>2</sub>.

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