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Measuring propionyl-CoA carboxylase activity in phytohemagglutinin stimulated lymphocytes using high performance liquid chromatography

Yu-Ning Liu [a], Tze-Tze Liu [a,b], Ya-Ling Fan [a], Dau-Ming Niu [c], Yin-Hsiu Chien [d], Yen-Yin Chou [e], Ni-Chung Lee [d], Kwang-Jen Hsiao [a,f], Yen-Hui Chiu [a,*]

[a] Department of Education and Research, Taipei City Hospital, Taipei, Taiwan
[b] VYM Genome Research Center, National Yang-Ming University, Taipei, Taiwan
[c] Department of Pediatrics, Taipei Veterans General Hospital, Taipei, Taiwan
[d] Department of Medical Genetics, National Taiwan University Hospital, Taipei, Taiwan
[e] Department of Pediatrics, National Cheng Kung University Hospital, Tainan, Taiwan
[f] Preventive Medicine Foundations, Taipei, Taiwan

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ABSTRACT

Background: Propionyl-CoA carboxylase (PCC) is a mitochondrial enzyme involved in the catabolism of several essential amino acids and odd chain fatty acids. Previous PCC assays have involved either a radiometric assay or have required mitochondria isolation and/or enzyme purification.
Methods: We developed an enzymatic method to analyze PCC activity in phytohemagglutinin (PHA) stimulated lymphocytes that involves high performance liquid chromatography.
Results: The method shows good linearity and sensitivity. PCC activity was unaffected even when lymphocytes were isolated and PHA stimulated after a whole blood sample had been stored at 4 °C for 5 days. This indicates that this method is suitable for analyzing samples from distant medical centers. The PCC activity of patients with propionic acidemia was found to be much lower than that of normal individuals and carriers. However, this PCC assay is significantly affected by the red blood cell contamination. In conclusion, this is a reliable method for performing PCC assays and only requires 0.5 to 1.0 ml of whole blood from newborns.
Conclusions: The PCC assay established in this study is useful for the confirmation of PA in individuals, and prenatal diagnosis and genetic counseling for the affected families.

1. Introduction

Propionyl-CoA carboxylase (PCC; EC 6.4.1.3) is a biotin-dependent mitochondrial enzyme that converts propionyl-CoA (PP-CoA) to methylmalonyl-CoA (MM-CoA), which is necessary for the catabolism of branched chain amino acids, odd numbered fatty acids, cholesterol, and various other metabolites [1]. PCC deficiency and the subsequent accumulation of abnormal compounds are associated with an autosomal recessive disease, propionic acidemia (PA; MIM 606054). Affected individuals may have episodes of metabolic acidosis with ketotic hyperglycinemia, hyperammonemia, or pancytopenia; these may lead to developmental delay, mental retardation and/or early death [1,2]. Early diagnosis and the appropriate limiting of the metabolic flux through the propionicogenic pathway are crucial to preventing life-threatening acute metabolic decompensation [3–5].

Tandem mass spectrometry has been used in neonatal screening programs in many countries to characterize patients with PA as having an elevation in C3-carnitine levels [3,4]. The organic acid profile of a urine sample is usually able to provide information allowing a confirmed diagnosis by the elevation of 3-hydroxypropionate and methyl citrate; however, differential diagnosis is essential to clearly separate PA from other metabolic conditions such as methylmalonic acidemia with non-classical phenotype [6,7], various conditions related to biotin processing and for screening healthy premature infants [8]. Other definitive diagnostic methods, such as genetic analysis and enzyme activity assays, have been used to construct a reliable mass screening system [9,10]. Unfortunately, it is sometimes difficult to distinguish mutations from polymorphisms, and some mutations, including large deletions and inversions, can only be detected by time-consuming and/or expensive molecular methods. Enzymatic assays, on the other hand, provide directly the information required for clinical diagnosis regardless of the genetic variation present. The gold standard for assaying PCC activity is a radiometric method; this measures the $^{14}CO_2$ release from sodium bicarbonate [11,12]. However, this radiometric assay has several

Abbreviations: FAD, flavin adenine dinucleotide; FBS, fetal bovine serum; IV-CoA, isovaleryl-CoA; IVD, isovaleryl-CoA dehydrogenase; MC-CoA, methylcrotonyl-CoA; MM-CoA, methylmalonyl-CoA; PA, propionic academia; PCC, propionyl-CoA carboxylase; PHA, phytohemagglutinin; PMS, phenazinemethosulfate; PP-CoA, propionyl-CoA.

* Corresponding author at: Department of Education and Research, Taipei City Hospital, No. 10, Sec. 4, Ren-Ai Rd., Taipei 10629, Taiwan.
E-mail addresses: yhchiu@ntu.edu.tw, A3360@tpech.gov.tw (Y.-H. Chiu).

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inconvenient features including being laborious due to its many required manipulations, need for laborious experimental set-up and waste disposal, as well as the limited applicability to high-throughput screening.

In view of the above problems associated with radiometric assays, several nonradiometric approaches have been reported in the literature for measuring enzyme activities related to the organic acidemias in biological samples [13–15]. Although a nonradiometric method has been developed to measure PCC enzyme activity in cultured cell lines and isolated tissue samples using a UPLC-MS/MS method [16], its procedure requires the isolation of mitochondria and enzyme separation, which are impractical in many clinical laboratories. Thus a convenient nonradiometric protocol for measuring PCC enzyme activity in biological samples is still lacking.

Leukocytes/lymphocytes have measurable amounts of PCC enzymes [16,17], but such specimens have many limitations. Firstly, PCC is unstable in whole blood if the samples are not properly preserved [17], which can lead to the recalling of patients and/or recollect of blood samples. Although adding acid–citrate–dextrose medium to whole blood does allow the survival of significant numbers of leukocytes for several days [17], the procedure is impractical in most clinical settings. Secondly, previous studies have suggested that 4 to 5 ml or more of whole blood is required for PCC assays using leukocytes/lymphocytes [17,18]. It is suggested that neonates who may have PA are subjected to clinical monitoring; however, it is difficult to obtain enough blood from those neonates for such a PCC assay. We therefore developed the use of phytohemagglutinin (PHA) stimulated lymphocytes as the target for a PCC assay. Additionally, when lymphocytes are cultured in 0.2 mg/l biotin contained RPMI-1640 medium, the process provides a significant amount of biotin and could be used for the differentially diagnosing PA and separating this disease from various biotin related metabolic disorders.

In this study, we developed an HPLC method to measure the amount of MM-CoA produced by the PCC enzyme from PHA stimulated lymphocytes. Our method showed good linearity and selectivity. Furthermore, samples from PA patients showed very low PCC activities compared to normal individuals and carriers. The PCC enzyme was found to be stable in whole blood that had been stored at 4 °C for 5 days followed by lymphocyte isolation and PHA stimulation. Moreover, PHA-stimulated lymphocytes could be obtained from 0.5 to 1 ml of neonatal blood and this is enough for the PCC assay. To our knowledge, this is the first nonradiometric method designed to assay PCC enzyme activity in clinical samples by MM-CoA quantification that does not require mitochondria extraction and/or protein purification.

2. Materials and methods

2.1. Subjects

Four PA families and 21 self-reported healthy individuals in Taiwan were recruited in this study. All patients were confirmed as having PCC deficiency by molecular analysis (Table 1) and by elevation of 3-hydroxypropionate and methylcitrate but not methylmalonate in urine using GC/MS/MS. The study was approved by the Institutional Review Board of the Taipei City Hospital, Taiwan. Informed consent forms were signed by all participants before the blood samples were taken.

Cases 1 and 2 in this study were the patients PA006 and PA008 from our previous studies [10]. Case 3 is a Taiwanese boy with normal physical development and moderate mental retardation (FIQ 46 at the age of 12 years old). Muscle weakness first occurred at 13 years old and spontaneously resolved. At 16 years old, general weakness appeared and lasted for one week without respiratory or gastrointestinal symptoms, fever, skin lesions, or central nervous system abnormalities. A decrease in muscle power and deep tendon reflex was noticed and he was diagnosed as axonal-type polyneuropathy. Elevations of creatinine kinase (427 U/l, normal range 55–170) and ammonia (60 μmol/l, normal range 9–33) were detected. During elective admission, creatine kinase and uric acid concentrations were found to be increased to 5610 U/l and 13.9 mg/dl (normal range 4.3–7.7), respectively. Hypertension, fever with leucopenia and thrombocytopenia, conscious change and spontaneous O_2 desaturation were also developed. The case was treated with steroid pulse therapy, plasmapheresis and endotracheal tube intubation/ventilation. Tandem mass spectrometry showed a great increase of C3-carnitine (64.14 μmol/l, cutoff: 5.14). Normal total carnitine 10.8 (6.3–11.6) but low free carnitine 1.29 (4.3–8.5) was also noticed. Urinary organic acid analysis showed elevations of 3-hydroxypropionic acid and 3-hydroxyvaleric acid, which suggested that he was a candidate for PA. On analysis, c.600 T > C and c.1087 T > C mutations in the *PCCB* gene were found. All his clinical and laboratory abnormalities disappeared within a few days when a low protein diet (1.5 g protein/kg/day) and oral supplement with L-carnitine (37.5 mg/kg/day) were implemented. However, epilepsy occurred in case 3 six months after. Although the patient was treated with antiepileptic drugs thereafter, his epilepsies were poorly controlled.

Case 4 was a Taiwanese boy without a family history of inherited metabolic disorders. The concentration of C3-carnitine (10.47 μmol/l, cut-off 4.8 μmol/l) was found to be high when a newborn screening test was carried out. 3-hydroxypropionate but not methylmalonate was increased when the dried blood spots from his first newborn screening were examined as a second-tier test, which suggested that he was a candidate for PA. Organic acid analysis of the subject's urine showed an elevation of 3-hydroxypropionate, succinate and glyoxime. Furthermore, c.1087 T > C and c.803del mutations of the *PCCB* gene were identified. On confirmation, the boy was treated by being fed a special milk formula and by oral supplementation with carnitine (50 mg/kg/day). The mild developmental delay and mild hypotonia at the age of nine months was noted.

2.2. Reagents

Histopaque-1077, PP-CoA, MM-CoA, isovaleryl CoA (IV-CoA), methylcrotonyl CoA (MC-CoA), adenosine triphosphate (ATP), flavin adenine dinucleotide (FAD) and phenazinemethosulfate (PMS) were

Table 1
PCC activity levels in various PA families.

Family		Mutations in the PCCB gene (paternal/maternal)	PCC activity* (pmol MM-CoA/min/mg protein)	IVD activity† (pmol MC-CoA/min/mg protein)
1	Case 1	c.1301C > T/c.1534C > T	5	107
2	Case 2	c.-4156_183 + 3713del/c.1301C > T	6	118
	Father	c.-4156_183 + 3713del	2376	153
	Mother	c.1301C > T	568	159
3	Case 3	c.600 T > A/c.1087 T > C	7	157
	Father	c.600 T > A	1084	128
	Mother	c.1087 T > C	1288	118
4	Case 4	c.1087 T > C/c.803del	6	124
	Father	c.1087 T > C	911	114
	Mother	c.803del	1208	177

* Range in normal individuals = 1321–2719 pmol MM-CoA/min/mg protein (n = 21).
† Range in normal individuals = 121–264 pmol MC-CoA/min/mg protein (n = 21).