NEVŞEHİR HACI BEKTAŞ VELİ ÜNİVERSİTESİ
Ara, bul.

NEVÜ Sağlık Bilimleri Dergisi
Cilt: 1 | Sayı: 1 | Aralık 2025

Akut lösemi alt tiplerinde eritrosit piruvat kinazının kinetik parametreleri etkileniyor mu?

Are kinetic parameters of erythrocyte pyruvate kinase being affected by acute leukemia subtypes

Abdullah Tuli[1], Hüseyin Özüsağlam[1], Hayri Levent Yılmaz[2], Ebru Dündar Yenilmez[1], Duygu Düzgünce[1], Atilla Tanyeli[3], Levent Kayrın[4]

[1]Department of Biochemistry, [2]Pediatric Emergency, [3]Pediatrics, Division of Pediatric Oncology, Faculty of Medicine, Çukurova University, Turkey. [4]Department of Biochemistry, Faculty of Medicine, University of Kyrenia, TRNC

tuliabd@cu.edu.tr

Anahtar Kelimeler
Eritrosit piruvat kinazı, kinetik, lösemi

Keywords
Erythrocyte pyruvate kinase, kinetics, leukemia

ÖZET

Eritrosit piruvat kinazı (ePK) eksikliğinin akut lösemi, malign lenfoma ve miyelodisplastik sendrom gibi hematolojik hastalıklardaki rolünü daha iyi anlayabilmek amacıyla, farklı akut lösemi tiplerinde ePK aktivite düzeyleri ve kinetik parametreleri araştırıldı.

Çalışma Çukurova Üniversitesi Tıp Fakültesi'nde yürütüldü ve 42 sağlıklı birey ile 53 lösemi hastasını kapsadı. Kontrol grubu ve akut lenfoblastik lösemi (ALL), nüks ALL (Relapse-ALL) ve akut non-lenfoblastik lösemi (ANLL) tanılı hastaların eritrosit hemolizatlarında yalnızca ePK düzeyi değil, aynı zamanda kinetik parametreler de ICSH tarafından önerilen yöntemle ölçüldü.

ePK aktivitesi (EU/gHb) sırasıyla kontrol, ALL, Relapse-ALL ve ANLL gruplarında 18.0±3.8, 14.9±7.0, 12.6±3.2 ve 11.7±3.0 olarak bulundu. Lösemi gruplarında ePK düzeyleri kontrol grubuna göre anlamlı derecede düşüktü ($p<0.05$). Ayrıca, lösemi gruplarındaki ePK kinetik parametreleri kontrol grubundan belirgin şekilde farklıydı. ANLL grubunda Vmax değeri kontrol grubuna göre anlamlı derecede düşük ($p<0.001$) iken, enzimin PEP'e olan afinitesi anlamlı olarak artmıştı ($p<0.05$). ADP için afinitenin ALL'de azaldığı ($p<0.001$), ANLL'de ise arttığı ($p<0.05$) saptandı.

Bu bulgular, ePK'nin aktivite ve kinetik parametrelerindeki değişikliklerin, löseminin gelişimi sırasında enzimin yapısında meydana gelen mutasyonlar veya kimyasal modifikasyonlardan kaynaklanabileceğini düşündürmektedir.

ABSTRACT

To enhance our understanding on the effect of ePK deficiency in various hematological disorders, including acute leukemia, malignant lymphoma, and myelodysplastic syndrome, we investigated the activity levels and kinetic parameters of ePK in different types of acute leukemias.

The study including 42 healthy subjects and 53 leukemic patients was performed in Çukurova University, Faculty of Medicine, Adana, Turkey. In this study, not only the level of ePK enzyme was determined but the kinetic parameters were also studied in the hemolysates of both the control and the patients with ALL, Relapse-ALL, and ANLL, using the method recommended by ICSH.

The mean and standard deviation values of ePK were 18.0±3.8, 14.9±7.0, 12.6±3.2, and 11.7±3.0 EU/gHb for control, ALL, Relapse-ALL, and ANLL, respectively. The results indicated that ePK levels were significantly lower in leukemias according to the control ($p<0.05$). Furthermore, we found that ePK kinetic parameters of leukemias displayed striking differences than those of the control. In ANLL, the V_{max} value was significantly lower than the control ($p<0.001$) but the affinity of the enzyme versus PEP showed significantly higher value ($p<0.05$). Meanwhile, the affinity of the enzyme versus ADP was significantly lower in ALL ($p<0.001$), but higher in ANLL ($p<0.05$) as per the control.

This suggested that these alterations in activity levels and kinetic parameters of ePK may be the result of mutations or chemical modifications happened in the structure throughout occurrence of leukemia.

Introduction

Erythrocyte pyruvate kinase (PK; ATP; Pyruvate 2-o-phosphotransferase EC 2.7.1.40) is an important key enzyme in the glycolytic pathway, which catalyzes the conversion of phosphoenolpyruvate to pyruvate with the generation of ATP (1). It has a molecular mass of approximately 200-240 kDa and is possessed of tetrameric structure with four identical or very similar subunits, each consisting of four domains (N-terminal, A, B and C) (2, 3). The active site resides between A and B domains, whereas the allosteric site is located in a pocket of the C domain (4). Four different PK isoenzymes (M_1, M_2, L, and R), encoded by two separate genes (PK-M and PK-LR) and expressed in a tissue-specific manner, are present in humans (5, 6). The PK-LR gene, located on chromosome 1 (1q21), codes for both PK-L (expressed in liver, renal cortex, and small intestine) (7) and PK-R (restricted to erythrocytes) through the use of alternate promoters (8).

The properties of erythrocyte pyruvate kinase (ePK), a rate controlling enzyme, showing a sigmoidal velocity curve with respect to its substrate phosphoenolpyruvate (PEP) (9) and having fructose-1,6-bisphosphate (FBP) as a positive allosteric effector are well-defined (10).

It is well known that deficiency of ePK is a cause of hereditary nonspherocytic hemolytic anemia, which was first defined in 1961 (2). Also the acquired ePK deficiency has been described in various hematological disorders, including acute leukemia (11), malignant lymphoma (12) and myelodysplastic syndrome (13) and the mechanism of such acquired enzyme deficiencies remains an object of discussion (14).

In this study, we investigated the levels of ePK and the kinetic parameters of healthy subjects and compared with those of acute lymphoblastic leukemia (ALL), relapse-acute lymphoblastic leukemia (Relapse-ALL) and acute nonlymphoblastic leukemia (ANLL). The main objective of determining ePK levels and kinetic parameters was to understand major mechanisms of acquired enzyme deficiencies in leukemias. Furthermore, prior to the kinetic assays we studied the best storage conditions for ePK.

MATERIALS AND METHODS

All of the chemicals used were analytical grade and obtained from BDH (England), Merck (Germany) or Sigma (U.S.A). All reagents were prepared by using glass-distilled water. The blood samples of leukemic patients were obtained from the Çukurova University, Faculty of Medicine Hospital, Pediatric Oncology and Hematology Department. Mainly four groups were included in this study: Group I, chosen as a control was consisted of 42 children, randomly selected among the healthy kindred of leukemic patients , Group II, III, and IV were comprised the ALL (n= 32), Relapse-ALL (n= 10) and ANLL (n= 11) after clinical and laboratory examinations. The subjects of all groups were between 3 and 14 years old and had no known ePK deficiency in the family history.

To understand effect of the storage conditions on enzyme of PK, venous blood samples taken from healthy subjects and leukemic patients were separately stored at +4 °C as whole blood and hemolysate, and at –20 °C, and –70 °C as hemolysate. The alterations in PK activity of the stored samples were measured at 5 days interval for a period of 45 days. The results were evaluated as percentage of the initial activity (activity of the first day was accepted as to be 100 %.).

After obtaining informed consent from parents/guardians, venous bloods (5 ml) of the subjects drawn into tubes with EDTA (1 mg/ml) were brought to the laboratory within 30 minutes of collection maintaining at +4 °C. The blood samples of leukemic patients, who have not received any chemotherapy and/or blood transfusion, were also included in the study. The specimens were stored at –70 °C in hemolysate form (as per the data obtained from storage assays) until experiments performed. To prepare the hemolysates, whole blood was centrifuged at 1000 g for 5 minutes and the erythrocytes were washed 5 times in cold 0.15 M NaCl. Hemolysates were consisted of 1 part of the erythrocyte suspensions free of leukocytes and platelets plus 9 parts of cold distilled water.

Pyruvate kinase activity was determined according to the standard methods of International Committee for Standardization in Haematology (ICSH) at 37 °C by following the decrease in absorbance at 340 nm, in a reaction coupled with lactate dehydrogenase with using hemolysates as an enzyme source (15). The assay mixture (600 µl) contained: 50 mM MOPS-NaOH at pH 7.5, 100 mM KCl, 10 mM 2-mercaptoethanol, 0.18 mM NADH, 2.4 units

of lactate dehydrogenase, 10 mM $MgCl_2$, 0.2 mM FBP, 1 mM ADP, 4 mM PEP (0.2-4 mM) and 15 µl hemolysate as the enzyme. Reactions were initiated by addition of the enzyme.

Kinetic studies in total of 32 specimens, on selected Control, ALL, Relapse-ALL, and ANLL within ±1 standard deviation of ePK activity, were assayed in the presence of FBP by using various concentrations of PEP and ADP under conditions identical to those described above. In all specimens, the enzyme activity was assayed at 6 different concentrations of PEP (4, 2, 1, 0.8, 0.6, 0.4, and 0.2 mM) and then ADP (2, 1, 0.8, 0.6, 0.4, and 0.2 mM). All measurements were performed in duplicate by using UV spectrophotometer (Shimadzu UV-260). V_{max} and K_m values were determined by plot of Lineweaver-Burk.

The enzyme activity was expressed in terms of Enzyme Units (µmoles of substrate converted/minute per gram of hemoglobin (EU/gHb)). Routine hematologic investigations were performed by electronic particle counter (Coulter T-890, Coulter Electronics Ltd.).

The results were given as the mean ± standard deviation. Statistical differences were determined by Kruskal-Wallis and Mann-Witney test. Besides, multiple comparisons of ePK activities and kinetic parameters of leukemic patients against control were done by using one-way ANOVA (Dunnett's method) (16) and p was considered significant at <0.05 level.

RESULTS

The activities of ePK of the samples stored at different temperatures are shown in Table 1. In all groups, ePK activity of the samples stored at +4 °C as whole blood decreased approximately 10% and 23% on the 5th and 10th days, respectively. When same samples were stored at +4 °C as hemolysate, the activities decreased about 7% and 13% on the 5th and 10th days, respectively. Moreover, considerable decreases in the enzyme activity of hemolysates were observed in the samples stored not only at +4 °C but also –20 °C. The hemolysates stored at –70 °C exhibited minimum decrease in enzyme activity as of 2% and 4% on the 5th and 10th days, respectively (Table 1). Due to minimum decrease in activity, the samples stored at –70 °C were used in the assays.

The statistical evaluation for levels of the ePK activity in different groups is shown in Figure 1 and Table 2. As shown in Figure 1, ePK value of the control was 18.0±3.8, as for the leukemias those were 14.9±7.0, 12.6±3.2 and 11.7±3.0 for ALL, Relapse-ALL and ANLL, respectively. Table 2 indicated that the ePK enzyme levels in leukemias except for ANLL were significantly lowered ($p<0.05$). Among the leukemic patients, the Relapse-ALL presented the most pronounced decrease ($p<0.001$). However, no significant differences were observed for ePK between the ALL, Relapse-ALL and ANLL patients.

When the initial velocity of the ePK was examined as a function of phosphoenolpyruvate concentration in the presence of FBP, a linear Lineweaver-Burk plot was obtained for all groups (Figure 2). However, when the kinetic parameters of ePK in the control were compared to those of the ALL, Relapse-ALL and ANLL ePK, some interesting results were inferred. With the exception of ANLL, the values of K_m (PEP) for Relapse-ALL and ALL were not changed relative to the control. In ANLL whereas the K_m (PEP) values were significantly decreased relative to the control, the affinity of enzyme versus the PEP was significantly increased (Figure 2, Table 3). When the same parameters were examined with various ADP concentrations, it appeared that the affinity of enzyme versus the ADP was significantly decreased in ALL but increased in Relapse-ALL and ANLL relative to the healthy subjects (Figure 3, Table 4).

DISCUSSION

It is well known that the energy requirement of the erythrocyte is only supplied in the form of ATP through the Embden-Meyerhof glycolytic pathway (5). Abnormalities in the ATP generating steps in this pathway cause the shortening of erythrocyte survival and autohemolysis, thereby generating hemolytic anemia (17).

Storage of whole blood for ePK determination at +4 °C was not satisfactory due to decrease in the enzyme activity up to 90% within first five days. For measurement of activity and kinetic studies of PK enzyme, we established the

best storage conditions of the medium to be –70 °C. Also, we believe that the samples should be analyzed within the first five days of collection.

Majority of ePK enzyme abnormalities described by several reports have been executed in acute leukemias. In this study, low ePK activity observed in leukemias seems to be acquired deficiency of ePK. The levels of ePK activity for all groups were in agreement with the other published studies (11, 12, 18, 19).

Furthermore, we determined that not only the activities but also the kinetic parameters were differed among leukemia subtypes. When the V_{max} and K_m values of ePK of the control were compared to the leukemias, significantly decreased V_{max} values were found for all leukemias, ANLL significantly low K_m value exhibited relative to the control. So, this explains the increased enzyme affinity towards PEP as being its substrate in ANLL.

The hypotheses proposed to explain the occurrence of acquired enzymopathies in acute leukemia may be as follows: (i) The presence of an inhibitor released by malignant cells (20). (ii) Partial reversion to a fetal form of erythropoiesis, including increased fetal hemoglobin (21). (iii) Mutations in the genetic material which may affect the production of erythrocyte enzyme (22, 23, 24). (iv) A number of quantitative and qualitative changes during gene expression (21, 25). (v) Posttranslational modification of enzymatic proteins that may lead to a decrease in activity or to a change in kinetic properties of red blood cell enzyme (11, 26, 27).

In the present study, we observed that, in ALL and Relapse-ALL, ePK with only decreased V_{max} values but unchanged K_m (PEP) values exhibited typical noncompetitive inhibition as per control, whereas, in ANLL, its Lineweaver-Burk plots constructed against both PEP and ADP demonstrated a parallel slope to that of control. It is important to recognize that a probable molecule exhibited inhibitory effect by binding to a modified site other than the enzyme's active site not only decreases the enzyme activity, but also increases the affinity towards its substrate PEP. The affinity of the enzyme towards ADP is decreased in high concentration of PEP in only ALL, but increased in Relapse-ALL. Observation of a kinetic behavior as uncompetitive inhibition in ANLL can be explained by the hypothesis mentioned at the first item above.

PK-R_1, one of two subtypes for PK-R, is predominantly situated in young erythroid cells as normoblast and reticulocyte while other subtype PK-R_2 is the major type in mature erythrocyte (28, 29). PK-R_1 as per PK-R_2 has a decreased substrate affinity and stability (30). In viewpoint of the foregoing data, we may conclude that decreased activity and kinetic behaviors of the enzyme are due to increased erythroid cells besides leukemic cells in leukemia (25, 31).

PEP is located in the cleft between A and B domains (10), whereas ADP is bound among A and C domains (2). While B domain of the enzyme displays flexible structure, A and C domains show more stable structure (3). Therefore, the enzyme's behaviors and values towards PEP alter at PK isoenzymes. However, K_m levels for ADP are very close to each other at its all isoenzymes (2, 32). In our study, significant difference in K_m (ADP) values between leukemias and control can be the result of a modification in the enzyme's structure which occurred during development of those diseases affecting the binding site for ADP; this structural modification does not influence binding site for PEP except ANLL. It is still difficult to answer the question whether this change of ePK affinity for ADP really reflects the altered enzyme function.

Moreover, the leukemic ePK enzymes becoming less stabile than the control at all storage temperatures could be given as the evidence of a probable structural modification at the site binding substrate of the enzyme, although there is no any definitive evidence (2, 23).

Numerous studies defined that ePK defects were as a consequence of either mutations on PKL gene which modify substrate binding sites of ePK (10, 23, 24) or translocations and chromosome breakpoints on 1q21 region (7, 22). Therefore, the defects influencing on binding of PEP and ADP to PK enzyme can give hints about causes of alterations on K_m and V_{max} determined in the study. Consequently, it is possible to deduce that the alterations on the enzyme are able to induce the mutagenesis and chemical modifications generating during leukemic development (21, 32), likewise decreased ePK activity can be acquired enzyme deficiency (14).

It is crucial to understand the metabolism of cancer cells and to determine key enzymes being targets of cytostatic treatments in important pathways. Hereby, it may be suggested that malignant cells should be distinguished from

normal cells qualitatively and quantitatively. The measurement of ePK activity can be used as an indicator to follow up and treat hematologic malignancies due to its being low in acute leukemias. It has not been known the underlying mechanism of acquired ePK deficiency in leukemias. However, we recommend that more studies are required to explain the accuracy of those interpretations and to utilize the value of ePK activity in leukemias.

Kaynakça

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