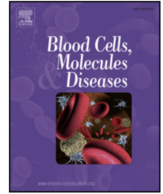
Blood Cells, Molecules and Diseases 64 (2017) 33–37

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd



# Creatine kinase in human erythrocytes: A genetic anomaly reveals presence of soluble brain-type isoform

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ARTICLE INFO

*Article history:*
Submitted 29 October 2016
Revised 14 March 2017
Accepted 17 March 2017
Available online 18 March 2017

Editor: Mohandas Narla

*Keywords:*
Brain-type creatine kinase
Cellular localization
Ectopic expression
Human erythrocytes

ABSTRACT

For maintaining energy homeostasis, creatine kinase (CK) is present at elevated levels in tissues with high and/or fluctuating energy requirements such as muscle, brain, and epithelia, while there is very few CK, if any, in peripheral blood cells. However, an ectopic expression of brain-type creatine kinase (BCK) has been reported for platelets and leukocytes in an autosomal dominant inherited anomaly named CKBE. Here we investigated CK in erythrocytes of CKBE individuals from eight unrelated families. The data revealed a varying but significant increase of CK activity in CKBE individuals as compared to controls, reaching an almost 800-fold increase in two CKBE individuals which also had increased erythrocyte creatine. Immunoblotting with highly specific antibodies confirmed that the expressed CK isoform is BCK. Cell fractionation evidenced soluble BCK, suggesting cytosolic and not membrane localization of erythrocyte CK as reported earlier. These results are discussed in the context of putative CK energy buffering and transfer functions in red blood cells.

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## 1. Introduction

Isoforms of creatine kinase (CK) are key players in cellular energy homeostasis by providing together with creatine and phosphocreatine an efficient energy buffering and transfer system [1–4]. High CK levels are therefore present in tissues and organs with high and/or fluctuating energy demands such as muscle, brain and epithelia, while this enzyme is much less abundant or even undetectable in some other cell types including hepatocytes and human blood cells. However, in erythrocytes of three probands from a family of Italian origin, unusually high CK activity in peripheral blood cells was described by Arnold et al. (1978) [5]. The CK isoform responsible for this activity was identified to be the brain-type CK (BCK) [6]. This ectopic expression of BCK (CKBE, MIM ID 123270) in blood cells is determined by a positive CK activity test in blood cells and normal CK activity in serum. It represents an autosomal dominant inherited anomaly with a frequency of about 1 out of 5000 persons. Most of the probands are in good health and do not show any hematologic disorder [6]. The mutation underlying the CKBE phenotype has been mapped to chromosome 14q32 where the BCK gene is located, but the molecular basis of this anomaly remains unknown [6]. CK in platelets and leukocytes has been analyzed in more detail in a recent study comparing CKBE probands of eight unrelated families or normal control individuals [6]. CK activity in probands was 50- and 7-fold higher in platelets and leukocytes, respectively, as compared to control individuals. Probands also showed significantly higher BCK mRNA levels that correlated with CK protein abundance.

Much less is known about CK in erythrocytes. Earlier data indicated that in contrast to other peripheral blood cells, human erythrocytes contain muscle-type CK (MCK) that is present in the membrane fraction [7], and this has been confirmed by first proteomic approaches [8]. Appreciable CK activities of 6.0 U/mg hemoglobin (Hb) and up to 37.3 U/mg Hb were also reported for rainbow trout and domestic chicken, respectively [9,10], and in the latter case again MCK was suggested as the principal erythrocyte CK. However, more recent proteomic studies do not identify any CK isoform in erythrocytes [11–14] and CK mRNA has neither been detected [15]. Thus, the questions remain whether red blood cells still express any CK activity, and if this is the case, which isoform and which putative function this would represent, and in particular how this activity would be affected in CKBE individuals.

*Abbreviation:* CK, creatine kinase; CK (BCK), brain-type; CK (MCK), muscle-type; CKBE, ectopic expression of brain-type creatine kinase; Ap5A, P1,P5-di(adenosine-5′)pentaphosphate; Hb, hemoglobin.

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http://dx.doi.org/10.1016/j.bcmd.2017.03.008
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In this study, we re-investigated the issue of erythrocyte CK by comparing CKBE and control probands [6]. We detected varying degrees of erythrocyte CK activity, but significantly higher in CKBE probands as compared to control individuals, and could identify this CK as a soluble, cytosolic form of BCK [4].

## 2. Subjects and methods

### *2.1. Subjects*

The subjects included in this study are identical to those described before [6]: 22 probands from eight unrelated families previously diagnosed with high levels of CK activity in peripheral blood cells and ten normal subjects (control group) composed of three normal individuals from these families and seven unrelated controls (healthy volunteers). Serum levels of CK activity were normal and comparable in CKBE probands and controls [6]. For blood collection, standard systems (S-Monovette®; Sarstedt, Nümbrecht, Germany) were used to obtain serum and anticoagulated (citrated) blood for isolation of erythrocytes.

### *2.2. Isolation of erythrocytes*

Citrated blood was centrifuged at 150*g* for 20 min. The upper layer representing the platelet-rich plasma and the intermediate layer (buffy coat) predominantly containing leukocytes were removed for other investigations. The residual lower layer significantly enriched for erythrocytes was stored at −80 °C and later used for hemolysis and for isolation of erythrocyte membranes.

In a pilot experiment we investigated the impact of leukocyte contamination on CK activity measured in erythrocyte membrane preparations. Blood samples were obtained from 3 healthy volunteer donors and processed as described above. Each sample was then splitted into two aliquots, one without leukocyte filtration and one with leukocyte filtration using leukocyte depletion filters (AutoStop® BC; Haemonetics, Munich, Germany). After automated leukocyte counting (Cell-Dyn 3700; Abbott, Wiesbaden, Germany) all samples were processed for determination of CK activity as described below. Leukocyte depletion was highly effective ($3.7 \pm 2.1 * 10^3$ WBC/μL versus $0.02 \pm 0.02 * 10^3$ WBC/μL; $p < 0.01$), whereas, CK activity was comparable ($0.22 \pm 0.03$ U/g Hb versus $0.20 \pm 0.06$ U/g Hb; $p > 0.05$) in unfiltered and filtered samples. Thus, leukocyte contamination had no impact on CK activity in erythrocyte preparations used in this study.

### *2.3. Isolation of erythrocyte membranes*

After hemolysis of erythrocytes by dilution in water to obtain a final concentration of 6.66 g/L, samples were centrifuged at 20000*g* for 10 min at 4 °C. The pellet obtained was washed by addition of TRAP buffer and again centrifuged at 20000*g* for 10 min at 4 °C. The supernant was then removed and the pellet resuspended in TRAP buffer. All work was performed at 4 °C.

### *2.4. Creatine kinase activity*

CK activity in lysed erythrocytes (dilution 1/5 in water) was determined in the reverse reaction (phosphocreatine and ADP as substrates). First, it was measured by a spectrophotometric assay using a coupled enzyme system as modified by Schlattner et al. (2000) [16] from a previous method by Wallimann et al. (1977) [17]. Briefly, ATP production by CK at pH 7 and 30 °C was coupled by hexokinase (30 U/mL) and glucose-6-phosphate dehydrogenase (15 U/mL) to NADP reduction, using 2 mM ADP, 5 mM $MgCl_2$, 20 mM phosphocreatine, 40 mM D-glucose and 1 mM NADP in 0.1 M triethanolamine buffer pH 7. To eliminate adenylate kinase activity that is known to interfere with the assay, red blood cell lysates were preincubated with 0.1 mM P1,P5-di(adenosine-5′)pentaphosphate (Ap5A) for 5 min. The absorbance increase at 340 nm was measured. Second, CK activity was determined independently by a highly specific electrochemical assay using a pH-Stat instrument (Radiometer Copenhagen, Villeurbane, France). The CK reverse reaction uses a proton that is then titrated back by the instrument to keep pH at a constant level in the solution constant. The reaction was measured at pH 7.0 and 25 °C in KCl medium (7.5 mM KCl, 0.1 mM EGTA, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol) in the presence of 1 mM ADP. The CK reaction was initiated by adding 10 mM phosphocreatine, and acid consumption was measured [18]. CK activities were calculated from the changes in $\Delta A_{340}$ or acid consumption before and after phosphocreatine addition. The amount of enzyme activity required to reduce 1 μmol NADP per min has been defined as 1 U. To normalize CK activity to the amount of erythrocytes, hemoglobin concentration was determined by the Drabkin reagent method, in which ferricyanide oxidizes oxyhemoglobin to methemoglobin, and cyanide converts methemoglobin to cyanmethemoglobin with an aborbance maximum at 546 nm. CK activity was then expressed in Units per g of hemoglobin (U/g Hb).

### *2.5. Immunoblotting*

Erythrocyte proteins (equivalent to 200 μg hemoglobin per lane) were separated by SDS-PAGE (1.5 mm, 12%, 45 min at 200 V in migration buffer), transferred onto nitrocellulose membranes (HybondTM ECLTM version LRPND/99/5; 150 mA for 1h10 in transfer buffer) and visualized by Ponceau Red staining to confirm equal loading. Destained membranes were incubated with primary antibodies for 2 h at 25 °C in TBS, 0.05% Tween and 4% (w/v) skimmed milk powder, washed 3 times for 15 min in TBS, incubated with secondary antibody coupled to Horse Radish Peroxidase HRP, either anti-mouse (Pierce) or anti-rabbit (GE Healthcare), for 1 h at 25 °C (1/2000 dilution), and washed again 3 times as above. Peroxydase activity was revealed by a chemiluminescence assay (Amersham TM ECLTM prime Western Blotting Reagents). Primary antibodies (and dilutions) used: chicken anti-human BCK and MCK (1:1000) [19], mouse anti-human BCK monoclonal 21E10 (1:500) [20]; rabbit anti-human P-Ser6 BCK (1:1000) [21].

### *2.6. Enzymatic erythrocyte creatine determination*

For creatine determination in red blood cells, erythrocytes were lysed in water (dilution 1/5), centrifuged (10,000 g for 10 min, 4 °C) and the supernatant filtrated (3 kDa cut-off filter, Microcon YM 3, Amicon Bioseparations) by centrifugation (14,000*g* for 45 min, 4 °C) to obtain a clear deproteinated filtrate. Creatine concentration was determined in the filtrate by a coupled enzymatic assay. Here, Cr used by CK (50 U/mL) is coupled by pyruvate kinase (3 U/mL) and lactate dehydrogenase (20 U/mL) to NADH oxidation, using 1 mM ATP, 25 mM $MgCl_2$, 0,62 mM phosphoenolpyruvate and 0,22 mM NADH in 0.1 M triethanolamine buffer, pH 8.5, at 30 °C. The final decrease in NADH concentration as measured by the change in absorbance at 340 nm is proportional to the creatine content. Creatine concentrations (given in μM) are calculated as micromole/L of packed erythrocytes.

### *2.7. Statistical analysis*

The statistical distribution of data obtained for CK activity is presented in a box plot diagram. Statistical significance was evaluated by a nonparametric test (Mann-Whitney *U* Test) with $p \leq 0.02$ (*).

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