Developmental changes in erythrocyte Na⁺,K⁺-ATPase subunit abundance and enzyme activity in neonates

Barna Vasarhelyi, Tivadar Tulassay, Agota Ver, Mariann Dobos, Istvan Kocsis, Istvan Seri

Abstract

Aim—To study the relation between erythrocyte Na⁺,K⁺-ATPase subunit isoform composition, Na⁺,K⁺-ATPase activity, and cation pump function in preterm and term neonates.

Design—Erythrocyte Na⁺,K⁺-ATPase subunit isoform abundance, Na⁺,K⁺-ATPase activity, and cation pump function were studied in blood samples obtained from 56 preterm neonates of 28–32 weeks gestation (group 1), 58 preterm neonates of 33–36 weeks gestation (group 2), and 122 term neonates (group 3) during the first two postnatal days.

Results— α_1 isoform abundance was higher and β_2 isoform abundance was lower in group 1 than in group 3 ($p = 0.0002$). α_2 and β_1 isoform abundance did not change with maturation and there was no evidence for the presence of the α_3 isoform. Gestational age was inversely related to Na⁺,K⁺-ATPase activity ($p = 0.0001$) and directly related to intracellular Na⁺ concentration ($p = 0.0025$).

Conclusions—Expression of the α_1 and β_2 Na⁺,K⁺-ATPase subunit isoforms is developmentally regulated. The increased abundance of α_1 isoforms of immature neonates translates to increased ATPase activity. The lower intracellular Na⁺ concentration of immature neonates suggests that their erythrocyte Na⁺,K⁺-ATPase cation pump function may also be increased.

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Na⁺,K⁺-ATPase is the main regulator of Na⁺ and K⁺ homeostasis. In addition, the electrical and concentration gradients generated by the enzyme are essential in the secondary active transport of other ions and solutes, the regulation of cell volume, and the electrical excitability of contractile and neural cell membranes. Functionally active Na⁺,K⁺-ATPase consists of two α and two β subunits.¹ The α subunits perform cation transport and possess the cardiac glycoside binding property and ATPase activity of the enzyme. Four different isoforms (α_1 , α_2 , α_3 and α_4) have been described with different kinetic properties.^{2–3} The β subunits play a role in ensuring the appropriate orientation of the α subunits in the cell membrane⁴ and their presence is unique in the family of cation pumps to

Na⁺,K⁺-ATPase. β subunits do not possess enzyme activity nor are they required for the ATPase activity of the enzyme. However, Na⁺/K⁺ exchange is not realised without these subunits.⁴ Three different isoforms of the β subunits have been identified for Na⁺,K⁺-ATPase.⁴

Altered Na⁺,K⁺-ATPase cation pump function may be an important element in the pathophysiology of several disease processes,⁵ including the electrolyte abnormalities associated with the syndrome of non-oliguric hyperkalaemia of the preterm neonate.^{6–9} Development also appears to have an impact on enzyme activity and cation pump function, but the findings are scanty and somewhat contradictory.^{6–10–13} Most studies on erythrocyte Na⁺,K⁺-ATPase activity during development have shown higher enzyme activity^{6–10–11} and ouabain binding capacity¹² in the immature animal and neonate than in the mature newborn. However, a recent study found that cord blood erythrocyte Na⁺,K⁺-ATPase activity is lower in preterm than term neonates.¹³ In addition to these conflicting results, the developmentally regulated changes in the relation between isoform composition and enzyme function have also not been clarified. Therefore, in this study we describe the characteristics of erythrocyte Na⁺,K⁺-ATPase α_1 , α_2 , α_3 , β_1 and β_2 isoform abundance in preterm and term neonates during the first two postnatal days and relate these observations to the Na⁺,K⁺-ATPase activity and cation pump function of the enzyme, providing information for the first time on the structural-functional relation of erythrocyte Na⁺,K⁺-ATPase during development.

Patients and methods

PATIENT POPULATION

A total of 114 preterm neonates between 28 and 36 weeks gestation and 122 full term neonates between 37 and 42 weeks gestation were enrolled in the study during the first two postnatal days. For data analysis, the patients were divided into three major groups according to their maturity. Preterm neonates with a gestational age between 28 and 32 weeks ($n = 56$; gestational age 30.5 (1.2) weeks; birth weight 1284 (271) g) and 33 and 36 weeks ($n = 58$; gestational age 34.3 (1.1) weeks; birth weight 2096 (458) g) formed groups 1 and 2 respectively, and the 122 term neonates gestational age 39.6 (0.9) weeks; birth weight 3247 (468) g comprised group 3. Owing to limited blood availability, not all of the patients in a given group contributed to all

Joint Research Program of the Hungarian Academy of Sciences and the 1st Department of Pediatrics, Semmelweis University School of Medicine, Budapest, Hungary
B Vasarhelyi

1st Department of Pediatrics, Semmelweis University School of Medicine, Budapest, Hungary
T Tulassay
M Dobos
I Kocsis

Department of Medical Chemistry, Molecular Biology and Pathophysiology, Semmelweis University School of Medicine
A Ver

Division of Neonatology, Department of Pediatrics, The Children's Hospital of Philadelphia and the University of Pennsylvania, Philadelphia, PA, USA
I Seri

Correspondence to:
Dr Seri, The Children's Hospital of Philadelphia, University of Pennsylvania, 34th Street and Civic Center Blvd, Philadelphia, PA 19104, USA
email: Seri@email.chop.edu

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of the measurements. Exclusion criteria included prenatal steroid treatment, postnatal administration of medications known to influence Na^+ , K^+ -ATPase activity and/or serum Na^+ and K^+ concentration such as dopamine, adrenaline (epinephrine), and diuretics, and the presence of acidosis (arterial $\text{pH} \leq 7.25$).

PREPARATION OF SAMPLES

Samples were collected when a routine blood draw was performed for a clinically indicated laboratory test. An additional 500 μl of heparinised blood was collected for the studies, and, after sedimentation of erythrocytes, the plasma was separated. Purified, haemoglobin-free erythrocyte membranes were prepared as described previously.^{14, 15} The protein content of the haemoglobin-free pellets was determined using bovine serum albumin as standard.¹⁶

WESTERN BLOT ANALYSIS

The α_1 , α_2 , α_3 , β_1 and β_2 subunit isoforms were studied by western blot analysis of purified erythrocyte membranes obtained from pooled blood samples as described previously.¹⁵ Rabbit anti-(rat Na^+ , K^+ -ATPase α_1 , α_2 , α_3 , β_1 and β_2 subunit isoform) antibodies and peroxidase conjugated goat anti-rabbit secondary antibodies were used, and the results of laser densitometry analysis were expressed as the percentage of the density in term neonates.¹⁵ The specificity of the antisera was verified on preparations of rat (heart, kidney, and brain) and human (kidney and small intestine) tissue homogenates (data not shown).

MEASUREMENT OF ERYTHROCYTE Na^+ , K^+ -ATPASE ACTIVITY

Enzyme activity was measured under $V_{\max}$ conditions for Na^+ , K^+ , and ATP and was calculated from the difference in NADH oxidation in the absence and presence of 1 mM ouabain as described previously.¹⁴ One unit of Na^+ , K^+ -ATPase represents 1 nmol ATP degraded/h/mg protein.

DETERMINATION OF INTRACELLULAR CATION LEVELS

Erythrocytes were washed, haemolysed, and diluted 30-fold for the determination of intracellular Na^+ concentration ($[\text{Na}^+]_{\text{ic}}$) and

50-fold for the measurement of intracellular K^+ concentration ($[\text{K}^+]_{\text{ic}}$). $[\text{Na}^+]_{\text{ic}}$ and $[\text{K}^+]_{\text{ic}}$ were measured with an atomic absorption spectrophotometer (932AA; GBC, Dandenong, Australia) in emission mode and are expressed in mmol/l erythrocyte volume.

ETHICAL APPROVAL

The study was approved by the institutional review board of the committee of protection of human subjects of the participating hospitals, and informed parental consent was obtained before enrollment.

STATISTICAL ANALYSIS

Data collected are given as means (SD) unless indicated otherwise. One factor analysis of variance (Fisher protected least squares difference test) and simple regression analysis were used where applicable. $p < 0.05$ was considered significant.

Results

Table 1 shows the relative abundance of the Na^+ , K^+ -ATPase subunit isoforms studied in the three groups of neonates. We did not find any evidence for the presence of the α_3 subunit isoform in neonatal erythrocytes, but α_1 , α_2 , β_1 and β_2 subunit isoforms were readily detected. The abundance of the α_2 and β_1 subunit isoforms remained unchanged during the studied period of human development. However, the abundance of the α_1 isoforms decreased while that of the β_2 isoforms increased with advancing gestational age. The $(\alpha_1 + \alpha_2)/(\beta_1 + \beta_2)$ ratio was 89% higher in the preterm neonates in group 1 than in the term neonates (group 3). However, as specific antisera to the α_4 and β_3 isoforms were not available at the time of the study, we cannot comment on the changes in the total α/β subunit ratio. Figure 1 shows a representative western blot developed by enhanced chemiluminescence illustrating the developmental regulation of erythrocyte Na^+ , K^+ -ATPase α_1 and β_2 subunit isoforms in nine characteristic samples.

Table 2 gives the Na^+ , K^+ -ATPase activity of the enzyme and $[\text{Na}^+]_{\text{ic}}$ and $[\text{K}^+]_{\text{ic}}$ in the three groups of neonates. Erythrocyte cell membrane Na^+ , K^+ -ATPase activity decreased with advancing gestational age, and there was an

Table 1 Relative abundance of α_1 , α_2 , α_3 , β_1 and β_2 subunit isoforms and the ratio of $(\alpha_1 + \alpha_2)/(\beta_1 + \beta_2)$ in purified erythrocyte membranes in groups 1, 2, and 3

	Group 1 (preterm neonates, 28–32 weeks)	Group 2 (preterm neonates, 33–36 weeks)	Group 3 (term neonates, 37–42 weeks)
α_1	176 (11)*	132 (23)†	100 (18)
α_2	100 (10)	108 (16)	100 (27)
α_3	0	0	0
$\alpha_1 + \alpha_2$	152 (10)*	122 (20)†	100 (37)
α_1/α_2	163 (10)*	113 (16)	100 (11)
β_1	94 (8)	93 (7)	100 (19)
β_2	51 (16)*	87 (32)	100 (12)
$\beta_1 + \beta_2$	80 (8)*	101 (12)	100 (14)
β_1/β_2	188 (17)*	125 (23)	100 (21)
$\alpha_1 + \alpha_2/\beta_1 + \beta_2$	189 (16)*	124 (31)	100 (20)

Purified erythrocyte membranes were obtained from pooled blood samples within each group, and western blot analysis ($n = 6$) was performed using subunit isoform specific antibodies. See the text for details. Results are means (SD) and are expressed as percentage of the findings in term neonates (group 3). The presence of α_3 subunit isoforms could not be documented.

* $p < 0.05$ v groups 2 and 3 (one factor analysis of variance; Fisher protected least squares difference test); † $p < 0.05$ v group 3 (one factor analysis of variance; Fisher protected least squares difference test).

Figure 1 Immunoblot analysis of Na^+ , K^+ -ATPase α_1 , α_2 , β_1 and β_2 subunit isoforms of neonatal erythrocytes. A representative western blot developed by enhanced chemiluminescence is shown obtained from pooled blood collected from patients in group 1 (gestational age 28–32 weeks; lanes 1–3), group 2 (gestational age 33–36 weeks; lanes 4–6), and group 3 (gestational age 37–42 weeks; lanes 7–9). Molecular mass markers run on the same gel were phosphorylase b (112 kDa) and ovalbumin (53 kDa). See the text for details.

Table 2 Na⁺,K⁺-ATPase activity in purified erythrocyte membranes and the intracellular sodium ([Na⁺]_{ic}) and potassium ([K⁺]_{ic}) concentrations in the three groups

	Group 1 (preterm neonates, 28–32 weeks)	Group 2 (preterm neonates, 33–36 weeks)	Group 3 (term neonates, 37–42 weeks)
Number of patients enrolled	56	58	122
Na ⁺ ,K ⁺ -ATPase (nmol ATP/h/mg protein)	624 (337)* (n=37)	481 (195)† (n=54)	404 (167) (n=113)
[Na ⁺] _{ic} (mmol/l)	7.09 (1.03) (n=24)	7.22 (1.53) (n=56)	8.04 (1.01)‡ (n=17)
[K ⁺] _{ic} (mmol/l)	94.0 (13.3) (n=16)	92.3 (11.0) (n=29)	89.8 (15.5) (n=18)

Values are means (SD).

*p < 0.05 v groups 2 and 3 (one factor analysis of variance; Fisher protected least squares difference test); †p < 0.05 v group 3 (one factor analysis of variance; Fisher protected least squares difference test); ‡p < 0.05 v groups 1 and 2 (one factor analysis of variance; Fisher protected least squares difference test); n = number of patients contributing to the given measurement. See the text for details.

inverse relation between Na⁺,K⁺-ATPase activity and gestational age ($y = 1219 - 20.6x$, $r = 0.345$, $p = 0.0001$; fig 2A) or birth weight ($p = 0.0011$; not shown). [Na⁺]_{ic} was significantly higher in group 3 than in groups 1 and 2 (table 2), and there was a significant direct relation between [Na⁺]_{ic} and gestational age ($y = 2.72 + 0.136x$, $r = 0.342$, $p = 0.0025$; fig 2B) or birth weight ($p = 0.0048$; not shown). There was no difference in [K⁺]_{ic} among the groups and no correlation could be found between [K⁺]_{ic} and gestational age and birth weight ($p = 0.143$ and 0.387 respectively; simple regression analysis, not shown).

Discussion

This study shows that erythrocyte Na⁺,K⁺-ATPase α_1 and β_2 subunit isoform expression is developmentally regulated in the neonate. We found a maturation dependent decrease in α_1

and an increase in β_2 subunit isoform abundance in neonates between 28 and 42 weeks of gestation. Our results also show that α_2 and β_1 subunit isoform abundance is not affected by maturation and that neonatal erythrocytes do not possess α_3 subunit isoforms. As the α_1 and β_1 subunit isoforms are the most commonly expressed isoforms in most of the tissues outside the nervous system,^{2–5} Na⁺,K⁺-ATPase activity and cation pump function are thought to be primarily determined by the abundance of these isoforms. In agreement with this notion, erythrocyte Na⁺,K⁺-ATPase activity was found to decrease with maturation, correlating with the changes in α_1 subunit abundance. Finally, the higher [Na⁺]_{ic} of mature neonates may represent a decrease in their erythrocyte Na⁺,K⁺-ATPase cation pump function commensurate with the developmentally regulated changes in ATPase activity. However, as unidirectional Na⁺ fluxes were not studied, developmentally regulated changes in the expression and/or function of Na⁺ leak pathways may have contributed to this finding.

Only limited information is available on the developmental regulation of erythrocyte Na⁺,K⁺-ATPase subunit isoform expression in man. We have recently shown that erythrocytes of term neonates express more α_1 subunit isoforms and have higher erythrocyte membrane Na⁺,K⁺-ATPase activity than those of children.¹⁵ In the present study, we extend this information to earlier stages of human development and provide convincing evidence that erythrocyte α_1 subunit isoform expression is developmentally regulated (table 1, fig 1). It is tempting to speculate that the upregulation of α_1 subunit expression during early development is, at least in part, related to the developmentally regulated high levels of endogenous inhibitors of Na⁺,K⁺-ATPase in the immature neonate.^{17,18} The fact that the Na⁺,K⁺-ATPase enzyme possessing the α_1 isoform is the most resistant to these endogenous digoxin-like substances^{1–2,5} makes this hypothesis even more attractive and suggests that there is a compensatory mechanism to maintain Na⁺,K⁺-ATPase function in the presence of higher concentrations of endogenous inhibitors.¹²

In agreement with our findings, most previous studies have also found higher erythrocyte Na⁺,K⁺-ATPase activity^{6,10,11} and ouabain binding capacity¹² in the immature animal and neonate than in the mature newborn. On the other hand, contrary to our present and the above mentioned previous findings,^{6,10–12} a recent study by Bistrizter *et al*¹³ found that cord blood erythrocyte ATPase activity is lower in preterm neonates of between 30 and 34 weeks of gestation than neonates of ≥ 35 weeks gestation. However, as Na⁺,K⁺-ATPase activity has been reported to increase after birth in preterm neonates,⁸ it is possible that the use of cord blood by Bistrizter *et al* affected their findings. In support of this hypothesis is the fact that they themselves reported a postnatal increase in erythrocyte Na⁺,K⁺-ATPase activity in their preterm neonates.¹³ Finally, our findings are strengthened by the enrollment of a larger patient population including more im-

Figure 2 Relation of gestational age to purified erythrocyte membrane Na⁺,K⁺-ATPase activity (A) and intracellular Na⁺ concentration (B). Erythrocyte membrane Na⁺,K⁺-ATPase activity is inversely related to gestational age ($y = 1218.7 - 20.6x$, $r = 0.345$; $p = 0.0001$; simple regression analysis), and intracellular Na⁺ concentration (mmol/l erythrocyte volume) relates directly to gestational age ($y = 2.715 + 0.136x$, $r = 0.342$; $p = 0.0025$; simple regression analysis). See the text for details.

mature preterm neonates and by the down-regulation of the α_1 subunit isoform in term neonates, suggesting the presence of an indirect relation between erythrocyte Na^+, K^+ -ATPase activity and gestational age.

We found lower $[\text{Na}^+]_{\text{ic}}$ in preterm neonates than in term neonates (table 2). In addition, there is also a direct relation between $[\text{Na}^+]_{\text{ic}}$ and gestational age (fig 2B) or birth weight. In agreement with these findings, other investigators have also reported a positive correlation between erythrocyte $[\text{Na}^+]_{\text{ic}}$ and gestational age.^{6 8 11 12} Thus it is tempting to speculate that, in addition to their increased ATPase activity, immature neonates also have a higher erythrocyte Na^+, K^+ -ATPase cation pump function. As for the $[\text{K}^+]_{\text{ic}}$, previous studies^{8 11} have also found that $[\text{K}^+]_{\text{ic}}$ does not necessarily change with maturation (table 2). However, because of the high $[\text{K}^+]_{\text{ic}}$ and the unique stoichiometry of the enzyme, it is generally believed that $[\text{K}^+]_{\text{ic}}$ is a less sensitive marker of Na^+, K^+ -ATPase cation pump function than $[\text{Na}^+]_{\text{ic}}$.¹⁰ Finally, as mentioned above, developmentally regulated changes in the expression and/or function of the Na^+ and K^+ leak pathways may have contributed to the changes in intracellular cation concentrations.

In summary, of the five erythrocyte Na^+, K^+ -ATPase subunit isoforms studied, only the α_1 and β_2 isoforms are developmentally regulated. The increased α_1 isoform abundance of preterm neonates is associated with enhanced erythrocyte Na^+, K^+ -ATPase activity and, on the basis of the findings for $[\text{Na}^+]_{\text{ic}}$, with an increased cation pump function of the enzyme. Finally, it is important to emphasise that the findings in erythrocytes may not represent the relation between Na^+, K^+ -ATPase subunit isoform composition and enzyme function in other tissues. Therefore caution should be exercised when speculating about the direct clinical significance of these findings.

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