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

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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—á subunit isoform abundance was higher and ß isoform abundance was lower in group 1 than in group 3 (p = 0.0002). a1 and ß isoform abundance did not change with maturation and there was no evidence for the presence of the a2 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 a1 and ß subunits, Na⁺,K⁺-ATPase subunit isoforms is developmentally regulated. The increased abundance of a1 isoforms of immature neonates translates to increased ATPase activity. The low Na/Na⁺ exchange is not realised without these subunits. Alterations in Na⁺,K⁺-ATPase cation pump function may be an important element in the pathophysiology of several disease processes, including the electrolyte abnormalities associated with the syndrome of non-oliguric hyperkalaemia of the preterm neonate. Development also appears to have an impact on enzyme activity and cation pump function, but the findings are scanty and somewhat contradictory.

Keywords: Na⁺,K⁺-ATPase activity; subunit isoforms; sodium concentration; preterm; erythrocyte; cation pump

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 a and two ß subunits. The ß subunits perform cation transport and possess the cardiac glycoside binding property and ATPase activity of the enzyme. Four different isoforms (a1, a2, ß1, and ß2) have been described with different kinetic properties. The ß subunits play a role in ensuring the appropriate orientation of the a subunits in the cell membrae and their presence is unique in the family of cation pumps to Na⁺,K⁺-ATPase. ß 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.

Three different isoforms of the ß subunits have been identified for Na⁺,K⁺-ATPase. Altered Na⁺,K⁺-ATPase cation pump function may be an important element in the pathophysiology of several disease processes, including the electrolyte abnormalities associated with the syndrome of non-oliguric hyperkalaemia of the preterm neonate.

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) were grouped 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...
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 pH < 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 The protein content of the haemoglobin-free pellets was determined using bovine serum albumin as standard.16

WESTERN BLOT ANALYSIS
The α₁, α₂, α₃, and β₁ subunit isoforms were studied by western blot analysis of purified erythrocyte membranes obtained from pooled blood samples as described previously.17 Rabbit anti-(rat Na⁺,K⁺-ATPase α₁, α₂, α₃, and β₁ 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 Vₘₐₓ 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 ([Na⁺]ic) and K⁺ concentration ([K⁺]ic) 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 α₁ and β₁ subunit isoforms in nine characteristic samples.

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 α₂ subunit isoform in neonatal erythrocytes, but α₁, α₂, β₁, and β₂ subunit isoforms were readily detected. The abundance of the α₁ and β₁ subunit isoforms remained unchanged during the studied period of human development. However, the abundance of the α₂ isoforms decreased while that of the β₂ isoforms increased with advancing gestational age. The (α₁+α₂)/(β₁+β₂) ratio was 89% higher in the preterm neonates in group 1 than in the term neonates (group 3). However, as specific antisera to the α₂ and β₁ isoforms were not available at the time of the study, we cannot comment on the changes in the total α/β subunit ratio.

Table 2 gives the Na⁺,K⁺-ATPase activity of the enzyme and [Na⁺]ic and [K⁺]ic in the three groups of neonates. Erythrocyte cell membrane Na⁺,K⁺-ATPase activity decreased with advancing gestational age, and there was a 50-fold increase in the Na⁺ concentration ([K⁺]c). [Na⁺]c, and [K⁺]c, were measured with an atomic absorption spectrophotometer (932AA; GBC, Dandenong, Australia) in emission mode and are expressed in mmol/l erythrocyte volume.

EVALUATION OF ERYTHROCYTE Na⁺,K⁺-ATPASE ACTIVITY
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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 α₂ subunit isoform in neonatal erythrocytes, but α₁, α₂, β₁, and β₂ subunit isoforms were readily detected. The abundance of the α₁ and β₁ subunit isoforms remained unchanged during the studied period of human development. However, the abundance of the α₂ isoforms decreased while that of the β₂ isoforms increased with advancing gestational age. The (α₁+α₂)/(β₁+β₂) ratio was 89% higher in the preterm neonates in group 1 than in the term neonates (group 3). However, as specific antisera to the α₂ and β₁ isoforms were not available at the time of the study, we cannot comment on the changes in the total α/β subunit ratio.

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Table 2  

<table>
<thead>
<tr>
<th>Number of patients enrolled</th>
<th>Na⁺,K⁺-ATPase (mmol ATP/h/mg protein)</th>
<th>[Na⁺]ic (mmol/l)</th>
<th>[K⁺]ic (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (preterm neonates, 28–32 weeks)</td>
<td>624 (337)*</td>
<td>7.09 (1.03)</td>
<td>94.0 (13.3)</td>
</tr>
<tr>
<td>Group 2 (preterm neonates, 33–36 weeks)</td>
<td>481 (195)†</td>
<td>7.22 (1.53)</td>
<td>92.3 (11.0)</td>
</tr>
<tr>
<td>Group 3 (term neonates, 37–42 weeks)</td>
<td>404 (167)‡</td>
<td>8.04 (1.01)‡</td>
<td>89.8 (15.5)</td>
</tr>
</tbody>
</table>

Values are means (SD).  

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

Discussion  

This study shows that erythrocyte Na⁺,K⁺-ATPase α₁ and β₁ subunit isoform expression is developmentally regulated in the neonate. We found a maturation dependent decrease in α₁ and an increase in β₁ subunit isoform abundance in neonates between 28 and 42 weeks of gestation. Our results also show that α₁ and β₁ subunit isoform abundance is not affected by maturation and that neonatal erythrocytes do not possess α₁ subunit isoforms. As the α₁ and β₁ subunit isoforms are the most commonly expressed isoforms in most of the tissues outside the nervous system,²⁻⁴ 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 α₁ 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 α₁ subunit isoforms and have higher erythrocyte membrane Na⁺,K⁺-ATPase activity than those of children.¹³ In the present study, we extend this information to earlier stages of human development and provide convincing evidence that erythrocyte α₁ subunit isoform expression is developmentally regulated (table 1, fig 1). It is tempting to speculate that the upregulation of α₁ 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.¹⁻⁴ The fact that the Na⁺,K⁺-ATPase enzyme possessing the α₁ isoform is the most resistant to these endogenous digoxin-like substances¹⁻² 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 activity¹⁰⁻¹¹ and ouabain binding capacity¹¹ 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,²⁻⁴ a recent study by Bistritzer et al¹³ 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,¹⁳ it is possible that the use of cord blood by Bistritzer 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.¹¹ Finally, our findings are strengthened by the enrollment of a larger patient population including more im-

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**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 linear regression analysis), and intracellular Na⁺ concentration (mmol/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 \( \alpha \) subunit isoform in term neonates, suggesting the presence of an indirect relation between erythrocyte Na\(^{+}\),K\(^{-}\)-ATPase activity and gestational age.

We found lower [Na\(^{+}\)]\(_i\) in preterm neonates than in term neonates (table 2). In addition, there is also a direct relation between [Na\(^{+}\)]\(_i\) and gestational age (fig 2B) or birth weight. In agreement with these findings, other investigators have also reported a positive correlation between erythrocyte [Na\(^{+}\)]\(_i\), and gestational age.\(^{6,8,11\text{-}12}\) 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 [K\(^{-}\)]\(_i\), previous studies\(^ {8,11\text{-}12}\) have also found that [K\(^{-}\)]\(_i\) does not necessarily change with maturation (table 2). However, because of the high [K\(^{-}\)]\(_i\) and the unique stoichiometry of the enzyme, it is generally believed that [K\(^{-}\)]\(_i\) is a less sensitive marker of Na\(^{+}\),K\(^{-}\)-ATPase cation pump function than [Na\(^{+}\)]\(_i\).\(^6\) 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 \( \alpha \) and \( \beta \) isoforms are developmentally regulated. The increased \( \alpha \) isoform abundance of preterm neonates is associated with enhanced erythrocyte Na\(^{+}\),K\(^{-}\)-ATPase activity and, on the basis of the findings for [Na\(^{+}\)]\(_i\), with an increased cation pump function of the enzyme. Finally, it is important to emphasize that the findings in erythrocytes may not represent the clinical significance of these findings.

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