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The influence of age on Na-K-ATP-ase activity in erythrocytes in fetal and newborn guinea pigs

K. G. Rosén, L. Sigström

Department of Physiology and Department of Pediatrics, University of Göteborg, Sweden

There is strong evidence that sodium-potassium activated adenosine-triphosphatase ($\text{Na}^+\text{-K}^+\text{-ATP-ase}$) located in the cell membranes mediates the active transport of sodium ions and potassium ions across the cell membranes. The degree of this extremely vital enzyme activity in different cell systems is linked to the function of each cell with high activities in cells with rapid shifts of sodium and potassium ions, as for instance in nerve cells. As ATP is hydrolyzed in this energy demanding "pumping" of sodium and potassium ions an adequate formation of ATP is important.

The dependancy of fetal metabolism on the anaerobic utilization of large stores of glycogen during hypoxia is well known [3, 4]. Thus, there is strong relationship between the preasphyxia levels of myocardial glycogen and the ability of the fetus to withstand asphyxia, measured as "time to last gasp" [3]. According to these findings it was also demonstrated that the myocardium of fetal guinea pigs was able to keep its ATP levels unaltered as long as there was glycogen left to be utilized during graded hypoxia [11]. In a previous study a high Na-K-ATP-ase activity has found to be related to a high glycolytic activity in erythrocytes [8]. Furthermore it has been demonstrated that anoxia leads to a marked increase in the rate of outflow of potassium ions in the diaphragm of adult rat, associated with a very high initial lactic acid production generated from the anaerobic utilization of glycogen [2]. Thus it appears to be

Curriculum vitae

K. G. ROSÉN, M.D. born in Stockholm 1948. Studied medicine at the University of Göteborg. Since 1971 working at the Dept. of Physiology and since 1976 at the Dept. of Pediatrics. In 1976 defended a thesis on alterations in the fetal ECG as a sign of fetal asphyxia.

of relevance to an organism with high glycolytic capacity also to have a parallel increase in sodium pump capacity.

Therefore it seemed to be of interest to elucidate the capacity of fetal and newborn animals to maintain an ion balance over the cell membrane as indicated by the $\text{Na}^+\text{-K}^+\text{-ATP-ase}$ activity in erythrocytes.

Material and methods

The experiments were conducted on 39 guinea pigs of different degrees of maturity. The gestational age was estimated from fetal weight using standard curves from DRAPER [5], and general appearance, the mature fetus looking like a miniature adult. The material was grouped as follows:

- 1: 8 immature fetuses weighing less than 50 g, gestational age 48–54 days

- 2: 8 mature fetuses, gestational age 60–65 days (term 65 days)
- 3: 8 newborn guinea pigs 2–3 days of age
- 4: 8 newborn guinea pigs 1 to 2 weeks of age
- 5: 7 pregnant guinea pigs

The guinea pig mother was anesthetized with ether as an induction and then tracheotomized. After cannulation of a carotid artery anesthesia was maintained with urethane (200 g/l) with a single dose of 0.4 g. Maternal blood sample was taken from the same catheter.

The fetus was exteriorized by a midline cesarean incision and blood was obtained from the umbilical cord by cutting it.

The newborn guinea-pig was anesthetized with ether and blood samples were obtained from a carotid artery.

Na-K-ATP-ase in erythrocytes was analyzed according to DODGE et al. [4] with modifications by WHAUN and OSKI [15] and further modification suggested by PHILLIPOT et al. [9]. EDTA blood was centrifuged at 600 G for 10 minutes. EVF was estimated and after that plasma, white blood cells and top layer of erythrocytes were sucked off. The remaining erythrocytes were washed twice in a solution containing 5 mM sodium phosphate per liter in 0.135 M saline, each time followed by centrifugation at 600 G and subsequent sucking off of the supernatant fluid. Redilution with phosphate solution to an EVF about 40%. Hemolysis was performed with 15 volumes of an ice cold solution with pH 8.0, containing 5 mM of TRIS (tris-hydroxy aminomethan) and 2 mM of EDTA per liter and after 15 minutes centrifugation was started at 15 000 G at 2°C for 30 minutes. The supernatant fluid was sucked off carefully and the remaining erythrocyte ghosts were brought to another tube and the same volume of the ice cold TRIS-EDTA solution as earlier was added to the ghosts and after 15 minutes followed by centrifugation at 15 000 G and 2°C for 30 minutes. The above procedure was repeated further once. The remaining erythrocyte membranes free from hemoglobin were frozen until incubation. After 60 minutes in a freezer the erythrocyte membranes were thawed and diluted with a solution containing 5 mM of TRIS per liter with pH 7.5 to a volume exceeding the original blood volume with about

25 per cent. 0.2 ml of this solution was added to tubes containing 0.8 ml of an incubation solution to a volume of 1 ml with a final concentration per liter of TRIS 15 mM, MgCl₂ 3.7 mM, KCl 5 mM, NaCT 43 mM, EDTA 0.25 mM and ATP 3.5 mM with or without ouabain 0.5 mM. Tubes containing all the compounds except magnesium were used as blanks to correct for non enzymatic breakdown of ATP. Tubes containing half the amount of erythrocyte membrane solution were prepared at the same time except for ouabain. All the procedures above were undertaken in a temperature of 6° and under strictly uniform circumstances from time to time. Incubation was started in a metabolic shaker containing water at 38°C. After 60 minutes the tubes were rapidly cooled with ice and 1 ml of 10% trichloroacetic acid was added to each tube. After centrifugation inorganic phosphate was determined according to SUBARROW and FISKE [6]. The absorbances were related to protein contents of erythrocyte membrane solution determined according to LOWRY [7].

2 Results

The mean values in each group of ouabain insensitive and ouabain sensitive adenosine triphosphatase (Na-K-ATP-ase) levels expressed as nanomoles inorganic phosphate per mg protein per hour are presented in Tab. I. The significance levels given in the text were calculated by using WILCOXON's ranking test.

Thus, the mean values of the levels of Na-K-ATP-ase (ouabain sensitive adenosine triphosphatase) in erythrocytes of immature fetuses is higher than in the other groups. During the first week after birth the levels are falling but rise again after about one week. When studying the ranges in the different groups there is some overlapping but the P-values between group I and the other groups show a significant difference ($p < 0.05$). There is also a significant difference between the mean values of group II and group III ($p < 0.05$) and between group III and group IV ($p < 0.05$). There is a relationship between immature low weighing fetuses and high Na-K-ATP-ase activity as is seen in Tab. I. One mature fetus and one guinea pig 9 days old, the levels of which exceeded 2000 nmol

Tab. I. Values of total ATP-ase and Na-K-ATP-ase expressed as nmoles inorganic phosphate per mg protein and hour.

No	Immature fetuses 8	Mature fetuses 8	Newborn 2-3 days old 8	Newborn 1-2 weeks old 8	Pregnant mothers 7
Weight grams					
Mean	35.6	98.3	89.4	113.5	1086
Range	(25.0-40.7)	(63.0-120)	(60.0-108.0)	(55.2-140.0)	(900-1100)
Total ATP-ase					
Mean	3251	2507	2003	2344	2038
SD	598	513	290	281	379
Range	2469-6111	1976-3593	1628-2200	1947-2824	1693-2701
Na-K-ATP-ase					
Mean	2179	1727	1369	1754	1488
SD	288	362	280	229	354
Range	1667-2492	1253-2449	913-1734	1398-2118	1167-2104
Per cent ouabain inhibition	67	68.9	68.3	74.8	73.0

inorganic phosphate per mg protein per hour both weighed less than the other animals of the groups II and IV. No relationship could be found when plotting the EVF of the original EDTA-blood specimen against the levels of Na-K-ATP-ase. No relationship could be found between the levels of Na-K-ATP-ase and the occurrence of coagulation in the original EDTA-blood specimen.

3 Discussion

This study was performed in order to investigate the "maximal" capacity of Na-K-ATP-ase activity in the erythrocyte membranes from guinea pigs with special regard to the influence of gestational and post partum age. **Statistically significant differences were obtained between the different groups of animals (Tab. I).** These alterations in Na-K-ATP-ase levels should not be due to the methodological error since the analyses were made in a strictly standardized way according to the above made description of the method. The random error of the method has been calculated in human erythrocytes and was found to be $\pm 3\%$. Thus the obtained alterations should be due to a real difference in the Na-K-ATP-ase activity of the different groups.

The changes in this enzyme level with a decrease after birth has previously been reported in the sheep [1]. The increase of ouabain inhibition with increasing age has also been reported. The total Na-K-ATP-ase levels in erythrocytes of fetuses and the total levels of the other groups of guinea pigs seem to have **similar relations as human erythrocytes from corresponding maturity groups.**

The pattern of alterations in the Na-K-ATP-ase level measured in erythrocytes from fetal and newborn guinea pigs ought to have some implications to the difference in metabolic situation to which the cells are subjected during this period of life, especially with regard to oxygen supply. Although this study only includes measurements made on erythrocyte membranes it is likely that changes occurring in these membranes will somewhat reflect those of other structures such as the myocardium. In view of previous findings [3, 13, 14, 11] which clearly demonstrate the dependency and capacity of the fetal myocardium on the anaerobic utilization of myocardial glycogen stores and the finding by PARKER and HOFFMAN [8] indicating a link between the glycolytic enzyme phosphoglycerokinase and Na-K-ATP-ase it can be assumed that an increase in the level of Na-K-ATP-

ase would be beneficial for a tissue with a high glycolytic capacity such as the fetal myocardium. The sequence of alterations found in the Na-K-ATP-ase levels with high levels measured in the immature fetus and a decrease towards birth are in parallel with the pattern of changes measured in the cardiac glycogen [3]. This parallelity seems to be of importance since glycogenolysis induces a liberation of potassium which is lost from the cell [5, 2]. This means a change of the potassium ion gradient over the cell membrane which has to be

minimized and restored by an increase in the activity of sodium pump. An increase in the Na-K-ATP-ase level reflects an increase in the capacity of this pump mechanism [10]. Thus a high sodium pump capacity is of importance in order to maintain the cellular integrity which is a main requisit for the function of different enzymes such as those included in the glycolytic pathway. Perhaps it is so that a high Na-K-ATP-ase capacity is involved in the superior ability of the fetus to survive anoxia.

Summary

One of the dominant factors behind the ability of fetal and newborn animals to sustain asphyxia is the anaerobic utilization of glycogen stored in the liver and myocardium. Glycogenolysis seems to induce a liberation of potassium ions leaking extracellularly and high capacity of the sodium pump might be essential in preserving the cellular integrity during asphyxia stress as well as during the phase of recovery where there ought to be a demand for an increase in sodium pump activity in order to restore the ion balance over the cell membrane. Thus, it seemed to be of interest to elucidate the capacity of fetal and newborn animals to maintain the ion balance over the cell membrane as indicated by the Na-K-ATP-ase activity in erythrocytes.

The material consisted of 8 immature fetuses, 8 mature fetuses, 8 newborn guinea pigs (2–3 days of age), 8 young guinea pigs (1–2 weeks of age) and 7 pregnant pigs. Blood samples were obtained from either the umbilical cord or a carotid artery and Na-K-ATP-ase activity was measured in the erythrocyte membranes according to DODGE et al. [4] with modifications. After sucking off of plasma and washing and centrifugation of the erythrocytes twice in PBS and after osmotic hemolysis and centrifugation at

15 000 G for 30 minutes three times in a solution with pH 8.0 containing 5 mmoles of TRIS and 2 mmoles of EDTA per liter, the hemoglobine free membranes were incubated in a solution containing TRIS, MgCl₂, KCl, NaCl, EDTA, ATP with and without ouabain for 60 minutes at 38°. Inorganic phosphate was measured and the activities were related to protein content of the stroma suspension.

The result showed a significant decrease of measured Na-K-ATP-ase activity with fetal age and the lowest values were found in newborns 1–2 days of age. After this age an increase in the enzyme activity was found, however the highest values always being found in the immature fetus.

Thus it appears as if in the fetal and neonatal period there is a decrease in sodium pump capacity of erythrocytes which seems to be in parallel with the ability to sustain asphyxia. Although the measurements were made in erythrocytes one can speculate whether these changes might give information about the pattern of alterations occurring in other tissues, e.g. the myocardium as well. Perhaps a high sodium pump capacity is involved in the superior ability of the fetus to survive anoxia.

Keywords: Erythrocytes, fetal guinea pig, newborn guinea pig, sodium pump activity.

Zusammenfassung

Der Einfluß des Alters fetaler und neugeborener Meerschweinchen auf die Na-K-ATPase Aktivität in Erythrozyten

Eine der vorherrschenden Faktoren für die Fähigkeit fetaler und neugeborener Tiere Sauerstoffmangel zu ertragen, ist die anaerobe Nutzung von Glykogen, das in Leber und Myokard gespeichert wird. Die Glykogenolyse scheint eine Freisetzung von Kalium-Ionen zu induzieren, die in den Extrazellulärraum gelangen. Zur Erhaltung der zellulären Unversehrtheit mag die große Leistungsfähigkeit der Natrium-Pumpe wesentlich sein, sowohl während der Belastung durch Sauerstoffmangel als auch während der Phase der Wiederherstellung, in der möglicherweise die Natrium-Pumpen-Aktivität zunimmt, um das Ionen-gleichgewicht an der Zellmembran wieder herzustellen. Daher scheint es von Interesse zu sein, die

Leistungsfähigkeit der fetalen und neugeborenen Tiere in Hinblick auf ihre Fähigkeit näher zu untersuchen, das Ionen-Gleichgewicht an der Zellmembran aufrecht zu erhalten. Dies wird durch Untersuchung der Na-K-ATPase Aktivität in den Erythrozyten beispielhaft gezeigt.

Das Material bestand aus 8 unreifen Feten, 8 reifen Feten, 8 neugeborenen Meerschweinchen (2–3 Tage alt), 8 jungen Meerschweinchen (1–2 Wochen alt) und 7 schwangeren Meerschweinchen.

Blutproben wurden entweder aus der Nabelschnur oder aus den Karotiden entnommen und die Na-K-ATPase Aktivität nach einem modifizierten Verfahren nach DODGE et al. [4] gemessen. Nach Absaugen von Plasma, Waschen und zweimaligem Zentrifugieren der Erythrozyten im PBS und nach osmotischer Hämolyse und Zentrifugieren bei 15 000 G 30 Minuten lang, je drei mal in einer Lösung mit einem pH von 8,0 und einer Konzen-

tration von 5 Mol TRIS und 2 Mol EDTA pro Liter, wurden die hämoglobinfreien Membranen mit einer Lösung, die TRIS, MgCl, KCl, NaCl, EDTA, ATP enthielt sowie mit und ohne Ouabain 60 Minuten lang bei einer Temperatur von 38° C inkubiert.

Anorganisches Phosphat wurde gemessen und die Aktivitäten wurden mit dem Proteingehalt der Stroma-Suspension in Verbindung gebracht.

Das Ergebnis zeigt einen signifikanten Abfall der gemessenen Na-KATP-ase Aktivität während des fetalen Alter. Die niedrigsten Werte wurden bei Neugeborenen im Alter von 1–2 Tagen gefunden. Nach diesem Alter wurde ein Anstieg in der Enzymaktivität gefunden, je-

doch wurden die höchsten Werte immer bei unreifen Feten gefunden.

Es scheint, daß in der fetalen und Neugeborenen-Periode eine Abnahme der Natrium-Pumpen-Kapazität der Erythrozyten vorliegt, die mit der verminderten Fähigkeit einhergeht, Sauerstoffmangel zu ertragen. Zwar werden die Messungen an Erythrozyten vorgenommen, jedoch kann man daraus schließen, daß die Veränderungen beispielhaft Auskunft geben über Veränderungen in anderen Geweben, z. B. auch im Myokard. Vielleicht hängt die hohe Natrium-Pumpen-Kapazität mit der überlegenen Fähigkeit des Feten zusammen, Sauerstoffmangel zu überleben.

Schlüsselworte: Erythrozyten, Fet, Meerschweinchen, Neugeborenes, Natrium-Pumpen-Aktivität.

Résumé

Influence de l'âge sur l'activité de Na-K-ATP dans les érythrocytes des cobayes à l'état de fœtus et de nouveau-né

L'un des principaux facteurs qui permettent aux animaux à l'état fœtal et de nouveau-né de lutter contre l'asphyxie est l'utilisation anaérobie du glycogène accumulé dans le foie et le myocarde. Il semble que la glycogénolyse libère les ions de potassium en perte extracellulaire et que la capacité élevée de la pompe de sodium joue un rôle essentiel dans la préservation de l'intégrité cellulaire pendant le stress d'asphyxie de même que durant la phase de rétablissement où la pompe de sodium doit probablement redoubler d'activité pour rétablir l'équilibre ionique par la membrane cellulaire. En conséquence, il nous a paru intéressant d'analyser cette faculté des animaux à l'état fœtal et de nouveau-né à maintenir l'équilibre ionique par la membrane cellulaire en corrélation avec l'activité de Na-K-ATP dans les érythrocytes.

Le matériel de notre étude a porté sur 8 fœtus immatures, 8 fœtus matures, 8 cobayes nouveaux-nés (âgés de 2 à 3 jours), 8 cobayes jeunes (âgés de 1 à 2 semaines) et 7 cobayes gravides. Des spécimens sanguins ont été prélevés sur le cordon ombilical ou une artère carotide et l'activité de Na-K-ATP a été mesurée dans les membranes érythrocytaires selon DODGE et al. [4] avec modifications. Après

aspiration de plasma et double lavage et centrifugation des érythrocytes dans PBS, après triple hémolyse et centrifugation osmotique à 15 000^g pendant 30 min. dans une solution avec p.H. 8.0 contenant 5 mmoles de TRIS et 2 mmoles d'EDTA par litre, les membranes libérées de Hb ont été incubées dans une solution contenant TRIS, MgCl₂, KCl, NaCl, EDTA, ATP avec et sans ouabaine à 38° pendant 60 min. Le phosphate inorganique a été mesuré et les activités ont été rapportées à la teneur en protéine de la suspension de stroma.

Les résultats ont montré une diminution importante de l'activité mesurée de Na-K-ATP selon l'âge fœtal, les valeurs les plus basses ayant été observées chez les nouveaux-nés de 1 à 2 jours, après quoi l'activité enzymatique augmente bien que les valeurs les plus élevées ont toujours été relevées chez les fœtus immatures.

Il semble donc que pendant la période fœtale et néonatale, la capacité de pompe de sodium des érythrocytes diminue de façon parallèle à la faculté de lutte contre l'asphyxie. Bien que les mesures aient été seulement effectuées sur les érythrocytes, il n'est pas impossible que les changements observés témoignent aussi d'altérations analogues dans d'autres tissus tels que le myocarde. Peut-être la faculté plus grande du fœtus à surmonter l'anoxie dépend-elle aussi d'une capacité élevée de pompe de sodium.

Mots-clés: Activité de pompe de sodium, cobaye fœtal, cobaye nouveau-né, érythrocytes.

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K. G. Rosén M.D.
Dept. of Pediatrics
Östra Sjukhuset
S-416 85 Göteborg
Sweden