Mechanism of Red Cell 2,3-Diphosphoglycerate Increase in Neonatal Lambs

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The tenfold increase in red cell 2,3-diphosphoglycerate (DPG) concentration that occurs during the first 5 days of life in lambs is an important adaptation to extrauterine life. In lambs, DPG reduces hemoglobin oxygen affinity by the Bohr effect. Our data on 10 neonatal lambs suggest that the biochemical mechanism underlying this DPG increase involves the following: (1) a rise in plasma glucose from 40 to 100 mg/dl in the first 48 hr of life, which allows for increased glucose consumption in the highly glucose-permeable neonatal RBC; (2) a transitory rise in blood pH begins at birth, peaks at about 20 hr, and falls slightly; (3) the pH increase coincides with a threefold increase in RBC 2,3-diphosphoglycerate mutase (GAPD), enolase (En), and pyruvate kinase (PK) in fetal red cells.

A dramatic rise in red cell 2,3-diphosphoglycerate (DPG) levels occurs in the first 5 days of life in the lamb. In sheep, unlike in man, DPG does not bind appreciably to either adult or fetal hemoglobin, but instead acts as an anion to decrease intracellular pH and thereby decrease hemoglobin oxygen affinity by the Bohr effect. Fetal sheep hemoglobin has intrinsically high oxygen affinity, and adult sheep hemoglobin has intrinsically low oxygen affinity. Previous investigations would suggest the following sequence of events for achieving appropriate hemoglobin oxygen affinity in the postnatal period. At birth, a tenfold rise in DPG levels begins, which produces a concomitant decrease in intracellular pH and an increase in P50 by the Bohr effect. By day 5 or 6, adult P50 values are achieved and are maintained thereafter. At about day 5 of life, DPG levels begin to fall and reach the very low adult levels by 50 days of age. This decrease in DPG parallels the disappearance of fetal hemoglobin from the circulation. Therefore, the postnatal rise in DPG levels is now generally accepted to be a compensatory mechanism to reduce the hemoglobin oxygen affinity of newborn sheep blood until red cells with adequate amounts of adult sheep hemoglobin are present.

While a number of studies have dealt with the physiologic role of the DPG rise, none has investigated the factors that contribute to this increase in red cell DPG. The underlying mechanism is of fundamental interest because it produces increases of an unusual magnitude. DPG increases from about 2.5 µmole/g Hb to 24 µmole/g Hb in the first 5 days of life. The highest DPG obtained by chronic bleeding in adult sheep was only 1.6 µmole/g Hb compared to the 0.2 µmole/g Hb seen in nonanemic adult sheep. Also, human newborns show a comparatively small 25% increase, which occurs during the first 4 days after birth.

In an earlier study, we determined that 17 of 20 red cell enzymes are significantly elevated in fetal sheep red cells compared to adult red cells. However, since many red cell enzyme activities decrease as red cells age in the circulation, and because red cell mass is rapidly expanding near term, the mean red cell population of late gestational sheep is much younger than the reticulocyte count indicates. In a second study, adult red cell enzyme activity measurements over a wide range of reticulocyte counts showed that most of the activity increases in fetal cells could be explained by young red cell populations. However, the degree of increase in activity of 5 fetal enzymes would require red cells comparable to populations with more than 31% reticulocytes in adult sheep. It was judged, therefore, that the high activities of phosphofructokinase (PFK), glyceraldehyde-3-phosphate dehydrogenase (GAPD), 2,3-diphosphoglycerate mutase (2,3 DPGM), enolase (En), and pyruvate kinase (PK) in fetal red cells could not be explained by their young cell age. The activity of PK, however, decreases to adult levels by birth. Therefore, PFK, GAPD, 2,3 DPGM, and En would be reasonable candidates for involvement in the postnatal DPG rise. We thus undertook the present study of red cell glycolytic interme-
DPG levels to characterize specifically the biochemical mechanism responsible for the rapid rise in red cell DPG in neonatal lambs.

MATERIALS AND METHODS

Adult, fetal, and newborn sheep of the Rambouillet-Columbia breed were used. Animals included 5 ewes that were 7 days postpartum and 13 chronically catheterized fetal sheep. Fetal catheters were implanted in pregnant ewes between 105 and 120 days of gestation using methods previously described. Blood samples were drawn at 111–125 days of gestation. Normal gestation is 149–150 days. No samples were obtained until 5 days postsurgery.

Two groups of spontaneously delivered lambs were bled by jugular venipuncture. The first group of 7 were bled on days 1, 3, 5, and 7 of life and thereafter less regularly. The second group of 4 lambs was bled between 3 and 6 times in the 48 hr after birth.

Six milliliters of whole blood were drawn into syringes containing heparin as anticoagulant. Five milliliters were immediately added to 10 ml of 0.6 M ice-cold perchloric acid for determination of glycolytic intermediates. After vigorous shaking and 20 min on ice, samples were frozen at −70°C until assay. The second group of lambs was bled only 3 ml per sample, and 1 ml was precipitated with 2 ml of perchloric acid. Whole blood hemoglobins and packed cell volumes were determined by standard methods.

Blood pH was determined using a Radiometer IL blood gas analyzer. Glycolytic intermediate levels were determined according to Minakami et al., except that levels of DPG and ATP were measured using the Sigma Kits 35-UV and 366-UV, respectively (Sigma Chemical, St. Louis, MO). Inorganic phosphate levels were determined on plasma samples precipitated with trichloroacetic acid and assayed according to a modification of the method of Fiske and Subbarow.

Glucose Consumption

Preliminary experiments of glucose utilization by lamb red cells were carried out in two ways. First, whole blood glucose was augmented by 100 mg/dl glucose using a solution of 1 g/dl glucose in 0.24 M NaCl, and samples were incubated in an open vessel placed in a 37°C shaking water bath for 4 hr. Samples were taken every 30 min after thorough mixing. Second, whole blood was passed through a mixture of α-cellulose and microcrystalline cellulose according to Beutler to remove white cells and platelets. Cells were washed twice with 0.24 M NaCl, then washed a third time in Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, made with 0.24 M NaCl, and finally suspended to a 20% hematocrit. Each sample was divided into 3 aliquots and glucose added to concentrations of 20, 50, or 100 mg/dl. Incubations were carried out as described above, and every 30 min, 0.2-ml samples were removed and added to 0.4 ml of 0.6 M ice-cold perchloric acid, frozen at −70°C, and assayed according to Noble et al.

Statistics

Data are presented as mean ± 1 standard deviation (SD) throughout. Unless otherwise stated, results were analyzed using Student’s t tests.

RESULTS

Glycolytic Intermediate Levels

The results of DPG determinations on the 11 lambs during the course of study are shown individually in Fig. 1. The magnitude of the DPG increase is apparent. Plasma glucose determinations on plasma from the 4 lambs in group 2 indicate that there is an increase from about 40 mg/dl at birth to about 100 mg/dl in the first 24 hr of life.

Table 1 gives the means and standard deviations of glycolytic intermediate levels from 5 maternal sheep. These data serve as the control data in Fig. 2, where the mean fetal and lamb intermediate values are expressed as percentages of mean maternal values. Intermediates are arranged in order of the glycolytic pathway as a crossover plot. The fetal values, shown by diamonds in Fig. 2, are quite similar to maternal
values, although DPG levels are significantly ($p < 0.001$) higher in fetal red cells, as are ATP levels ($p < 0.01$). By the first day of life, red cell intermediates change dramatically, as is shown by the closed circles in Fig. 2. The first four glycolytic intermediates shown, G6P, F6P, FDP and DHAP, as well as AMP (not shown) are significantly increased ($p < 0.025$). Although DPG is somewhat increased, none of the intermediates after DHAP is significantly different in fetal sheep and day 1 lambs. The fact that F6P levels are slightly increased, but FDP and DHAP levels are markedly elevated, suggests that the enzyme that converts F6P to FDP, namely PFK, is activated in day 1 lamb compared to fetal and maternal red cells. The finding that DPG is not significantly elevated at day 1 suggests a relative metabolic block after DHAP and before DPG, i.e., at the enzymatic step catalyzed by GAPD.

Intermediate data for day 3 lambs are shown in Fig. 2 as closed triangles. Here, two clear crossover points are seen when day 3 lambs are compared to day 1 lambs. Levels of F6P are higher and levels of FDP are lower in day 3 lambs compared to day 1 lambs, indicating a relative decrease in PFK activity. By day 3, DHAP levels are considerably decreased while DPG levels are greatly increased, suggesting relief of the metabolic block hypothesized at the GAPD step on day 1. Day 5 intermediate levels (closed squares, Fig. 2) reveal a further increase in DPG levels and decreases in preceding intermediates. The peak of the DPG increase occurs on about day 5 (Fig. 1). Mean fetal, day 1, and day 5 lamb ATP levels were significantly higher ($p < 0.05$) than maternal values. Levels of day 1 lamb AMP were $236\%$ of maternal values and were significantly higher ($p < 0.05$) than all other groups measured. No significant changes in NAD levels were seen.

These data strongly suggest changes in the activities in vivo of enzymes PFK and GAPD in the neonatal period. The activity of PFK is highly modified by external factors, one of which is pH. Figure 3 shows pH and FDP data from the second group of lambs, where venipuncture was frequently performed in the immediate postnatal period. There is a transitory rise in both blood pH and FDP. Inorganic phosphate is a substrate of the enzyme GAPD, and its activity has been shown to be limited or increased in a number of diseases where plasma phosphate is low or high. Figure 4 shows that a rise in plasma phosphate after birth is

![Diagram](attachment:Diagram.png)

**Fig. 2.** Red cell glycolytic intermediate levels in fetal (●), day 1 (▲), day 3 (△), and day 5 (□) lambs expressed as percentages of the mean values for 5 maternal sheep. The glycolytic pathway is shown below.

![Diagram](attachment:Diagram2.png)

**Fig. 3.** Whole blood pH (A) and red cell fructose-1,6-diphosphate (FDP) (B) levels in 4 newborn lambs. Similar symbols are for the same lamb. The abscissa represents time in hours after birth.
closely paralleled by the rise in DPG levels. Using these data, a highly significant ($p < 0.001$) correlation ($r = 0.87$) is seen between DPG and plasma phosphate.

Glucose Consumption

The mean whole blood glucose consumption for 10 lambs aged 3 hr to 24 days was $0.23 \pm 0.05$ mg glucose/ml red cells/hr. Within the limits of this sample size and the sensitivity of the procedure, no relationship with age was apparent.

The effect of glucose concentration on the rate of glucose consumption was examined using 9 blood samples from lambs of the following ages: 6, 17, and 24 hr, and 1, 3, 5, 11, 13, and 24 days. Glucose consumption in KRB averaged $0.40 \pm 0.22$, $0.31 \pm 0.11$, and $0.28 \pm 0.09$ mg glucose/ml red cells/hr for samples incubated with glucose concentrations of 100 mg/dl, 50 mg/dl, or 20 mg/dl, respectively. Paired t tests revealed that glucose consumption in KRB containing 100 mg/dl glucose is significantly ($p < 0.05$) higher than in KRB containing 20 mg/dl glucose. An apparent relationship between the age of the lamb and the difference between glucose consumption at 100 mg/dl and 20 mg/dl was tested using a rank correlation test. The correlation coefficient ($r_s = 0.71$) was significant ($p < 0.025$), indicating that the effect of high glucose levels on glucose consumption is greater in red cells from young lambs than in red cells from older lambs.

DISCUSSION

Our intermediate data suggest that dramatic changes occur in lamb red cell metabolism during the first 5 days of life. Data presented in Fig. 1 on neonatal changes in DPG levels are very similar to those published by Bard et al. and clearly show the tenfold increase that occurs in the first 5 days of life. The extensive intermediate data presented in Fig. 2 suggest a sequence of events that leads to the DPG rise. It appears that between late gestation and day 1 of life, the enzyme PFK is activated, probably as a result of increased plasma pH. The data in Fig. 3 suggest that the increases in both the levels of PFK product, FDP, and whole blood pH begin at or very close to birth. The dramatic increases in FDP and DHAP, which occur in day-1 lamb red cells, do not produce increases in DPG or 3PG levels, as would be expected in the absence of a block after DHAP. Our data suggest that this block may result from insufficient inorganic phosphate, a substrate of the GAPD step. As inorganic phosphate levels increase (Fig. 4), the block is relieved and DPG levels rise. Interestingly, one would expect that 3PG levels would rise somewhat after relief of the block at the GAPD step. The fact that 3PG does not rise significantly between days 1 and 3 suggests that the DPG synthetase enzyme, 2,3DPGM, competes very successfully with phosphoglycerate mutase for their mutual substrate 1,3DPG. These findings are consistent with results of our two earlier studies. Fetal sheep red cells contain very high activities of 2,3DPGM. The mean activity for this enzyme in four lambs aged 1 day was $0.41 U/10^{10}$ RBC, whereas the adult mean activity was $0.04 U/10^{10}$ RBC for 8 ewes. Also, chronic phlebotomy of adult sheep produced no increase in 2,3DPGM activity, suggesting that the young red cell populations of newborn sheep could not explain the high 2,3DPGM activities in fetal sheep. It appears likely, therefore, that either these high activity levels are due to a fetal 2,3DPGM isozyme, or the adult enzyme is present in greater quantities.

Our results confirm those of others that plasma glucose levels increase about 2.5-fold during the first 24 hr of life. The KRB incubation experiments suggest two possibilities. First, in this age range, there is a difference in glucose consumption at different extracellular glucose concentrations: a higher extracellular glucose results in higher glucose consumption. Thus, the 2.5-fold increase in plasma glucose in the first day of life may allow increased red cell glucose utilization, although our limited data on whole blood glucose consumption do not prove this. Second, this relationship between extracellular glucose concentration and glucose consumption decreases with the animals' age. This result is not surprising in light of glucose uptake...
and permeability studies showing a high correlation ($r = 0.995$) between glucose transport and fetal red cell count in newborn sheep. Mooney and Young found that glucose permeability was decreased 12-fold in red cells appearing immediately after birth compared to fetal lamb red cells.

In conclusion, these data suggest that the following sequence of events contributes to the postnatal rise in DPG in the lamb. First, the concentration of plasma glucose more than doubles in the first 48 hr, supplying substrate for the highly permeable fetal red cell. Second, a transitory rise in blood pH produces an activation of red cell PFK, but because of insufficient plasma phosphate, a substrate of the GAPD step, the DPG increase is limited. Third, as the plasma concentration of phosphate increases, GAPD is activated and DPG levels rise dramatically. Finally, the high activities of PFK, GAPD, and DPGM observed in fetal red cells are probably necessary for such dramatic metabolic changes.

ACKNOWLEDGMENT

The authors are grateful to Tom Togioka and Lisa Stephens for their excellent technical assistance and to Evie Larson for help in preparation of the manuscript.

REFERENCES

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