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SHORT COMMUNICATION

CHANGES IN RED CELL ATP CONCENTRATION AND OXYGEN-AFFINITY FOLLOWING BIRTH IN THE NEONATAL GARTER SNAKE *THAMNOPHIS ELEGANS*

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The neonate of viviparous reptiles is clearly precocial relative to the mammalian neonate and shows a rapid transition to independent life following parturition. This suggests major physiological changes occur quickly at, and just after, parturition. In this study, we examined the temporal changes that occur in the respiratory properties of red blood cells in the garter snake *Thamnophis elegans* at birth.

The red cells of the *T. elegans* fetus have a higher affinity for oxygen than do maternal cells (Ingermann *et al.* 1991). This difference in affinities appears to be due to the presence of lower concentrations of nucleoside triphosphates (NTP, principally ATP) in fetal red cells (Berner and Ingermann, 1988). (These organic phosphates lower oxygen affinity by allosterically modulating hemoglobin function.) Oxygen availability probably influences red cell NTP concentrations *via* oxidative phosphorylation in at least some of the lower vertebrates (Wood and Johansen, 1972; Greaney and Powers, 1977, 1978; Ingermann *et al.* 1983). Therefore, it is possible that, at parturition, the red cells of the neonatal snake respond to increased oxygen availability by increasing red cell NTP levels. This could have the consequence of quickly lowering oxygen affinity to, or towards, adult values.

Although red cell NTP levels rise following birth in the garter snake, the qualitative changes that take place during birth may be more complex. The mammalian fetus probably does not become hypoxic during parturition until after birth, when the umbilical cord is severed (Comline and Silver, 1972*a*,*b*; Adamson *et al.* 1987). The fetal garter snake, however, is not attached to the mother by a comparable umbilical cord (Hoffman, 1970) and it may experience hypoxia during parturition as it is detached from its developmental position in the uterus and is

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moved towards the cloaca. If the fetus experiences hypoxia during this process, one might expect to see a transient decrease in red cell NTP concentrations at birth.

Consequently, we have measured the change in red cell NTP concentration as a function of neonatal age and have measured oxygen affinity at two times following birth. We have also examined the relationship between oxygen availability and NTP levels with neonatal red cells incubated *in vitro* with metabolic inhibitors.

Adult garter snakes were collected in early June in Latah Co., Idaho, and identified as *Thamnophis elegans* (Baird and Girard) according to Nussbaum *et al.* (1983). Adult snakes and neonates were kept on approximately natural photoperiods (14 h:10 h) L:D and at a temperature of 20°C with continuous access to water. Adults also had access to an electric heater and were fed beef heart and fish about once per week. One-month-old neonates were fed earthworms and fish once per week.

Adult animals were bled by heart puncture under ether anesthesia. To obtain red cells from neonates, they were rendered unconscious, decapitated and sectioned in buffer. Cells were then filtered with glass wool to eliminate larger debris. Snakes were bled into ice-cold, heparinized, bicarbonate-buffered saline (110 mmol l^{-1} NaCl, 1.9 mmol l^{-1} KCl, 1.1 mmol l^{-1} $CaCl_2$, 2.4 mmol l^{-1} $NaHCO_3$, pH 8.0). Red cells were washed three times in this saline by centrifugation at 1000 *g* for 5 min at 4°C. To establish the time course of the NTP change following birth, red cells were pooled from at least two individuals per time point, washed and resuspended, and extracted within 30 min of blood collection with an equal volume of ice-cold 12 % trichloroacetic acid (TCA). This mixture was centrifuged at 12 000 *g* for 5 min and the supernatant was analyzed for NTP concentration with an enzymatic assay kit (no. 366-UV) from Sigma Chemical Company (St Louis, MO). The hemoglobin concentration of the red cell suspension was determined spectrophotometrically using a millimolar heme extinction coefficient of 11.0 for cyanmethemoglobin at 540 nm. NTP concentrations are expressed as a molar concentration (calculated with the hematocrit of the red cell suspension prior to extraction with TCA) or as a ratio of mole NTP per mole hemoglobin tetramer. Mean corpuscular hemoglobin concentrations (MCHC) were calculated from hemoglobin concentrations and hematocrit values of the final red cell suspensions.

P_{50} measurements were made on red cells pooled from 2–5 individuals per litter. These measurements were made by the method of Tucker (1967) with a TC500 Tucker cell and model 781 oxygen meter from Strathkelvin Instruments (Glasgow, Scotland). Red cells were deoxygenated with nitrogen and reoxygenated with a nitrogen/compressed air mixture, as previously described (Ingermann *et al.* 1991). The pH 7.4 buffer used for these studies has also been described previously (Ingermann *et al.* 1991).

For *in vitro* incubations, neonatal red cells were obtained by pooling 5–15 individuals per single litter. Filtered, washed and pelleted red cells were resuspended in a modified Rugh's (1962) saline (150 mmol l^{-1} NaCl, 4 mmol l^{-1}

KCl, 2 mmol l^{-1} $CaCl_2$, 0.5 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} KH_2PO_4, 2 mmol l^{-1} Na_2HPO_4, 10 mmol l^{-1} D-glucose, pH 7.4). Red cell suspensions had hematocrit values of 4–10 % and incubations were conducted with occasional stirring (approx. 15 min intervals) at 25 °C. To assess the role of oxidative phosphorylation, red cells were incubated with either 100 μmol l^{-1} sodium cyanide or 500 μmol l^{-1} iodoacetate.

In all experiments, cells of individual neonates were pooled from each of N different litters. All data are presented as mean±S.D. Except where noted, statistical analyses were based on single factor analysis of variance (ANOVA) followed by the Tukey test (alpha=0.05) for equal or unequal sample sizes as appropriate (Zar, 1984).

As previously reported (Ingermann *et al.* 1991), fetal values for the red cell concentration of NTP and the molar ratio of NTP to tetrameric hemoglobin are 5.58±0.77 mmol l^{-1} and 1.69±0.32, N=11; adult male values are 8.20±0.86 mmol l^{-1} and 2.23±0.22, N=17, respectively. Red cell NTP levels changed rapidly after birth (Fig. 1). The earliest time after birth when the red cell NTP concentration or the NTP/hemoglobin ratio was not significantly different from the value of the adult male was at 6 h. MCHC values were significantly different (P<0.01) between fetal (3.25±0.32 mmol l^{-1}, N=9) and adult males (3.76±0.47 mmol l^{-1}, N=16) (t-test). However, although analysis of MCHC values by ANOVA indicated that a significant difference existed for the developmental groups examined, the Tukey test was unable to define which values were different. The P_{50} values of red cell suspensions from the fetus, 6 h neonate, 1 month neonate and adult male were 2.48±0.63, 3.59±0.37, 3.95±0.71, and 4.29±0.91 kPa, respectively (N=5, all) (1 kPa=7.50 mmHg). The P_{50} value for the red cell suspension of the adult male was significantly different from that for fetal

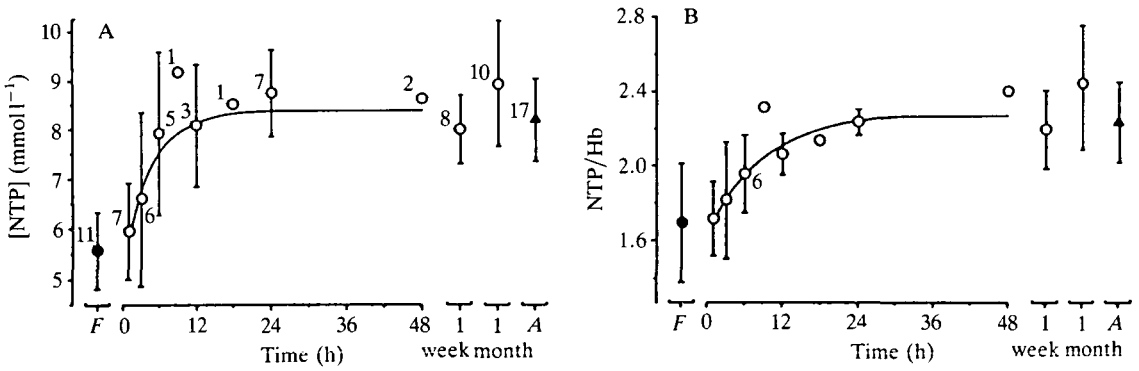

Fig. 1. Concentration of red cell NTP expressed as a molar concentration (A) and as a molar ratio of NTP to hemoglobin tetramer (B) expressed as a function of time after birth. F refers to the fetal value (●); A refers to the adult male value (▲). Small numbers adjacent to the data points refer to numbers of litters; unless otherwise indicated, numbers of litters per time point in A and B are the same. Data are mean±S.D. and curves were fitted by eye.

red cells; it was not different from values for red cells of the 6 h or 1 month neonate.

When incubated *in vitro*, neonatal red cells showed progressive declines in NTP levels but essentially no changes in hematocrit. However, cyanide and iodoacetate increased the rate of decline (Fig. 2). After 6 h at 25 °C, cyanide- and iodoacetate-treated cells contained 60.0±3.2 % (N=3) and 74.1±7.6 % (N=3), respectively, of control values of NTP.

The oxygen affinity of hemoglobin from the garter snake is modulated by ATP and maternal–fetal oxygen transfer in this animal appears to be facilitated by different maternal and fetal concentrations of ATP within the nucleated red cell (Berner and Ingermann, 1988). One possible advantage of this molecular strategy, in comparison with utilizing distinct fetal and adult hemoglobins to facilitate maternal–fetal oxygen transfer, is that the change in the functional properties of the blood from fetal to adult characteristics may occur very rapidly after parturition. Indeed, in mammals with distinct fetal hemoglobins, the replacement of fetal by adult hemoglobin may be completed one or more months after birth (Bard *et al.* 1972; Bard, 1975); in those mammals possessing different maternal and fetal concentrations of organic phosphate (2,3-diphosphoglycerate in mam-

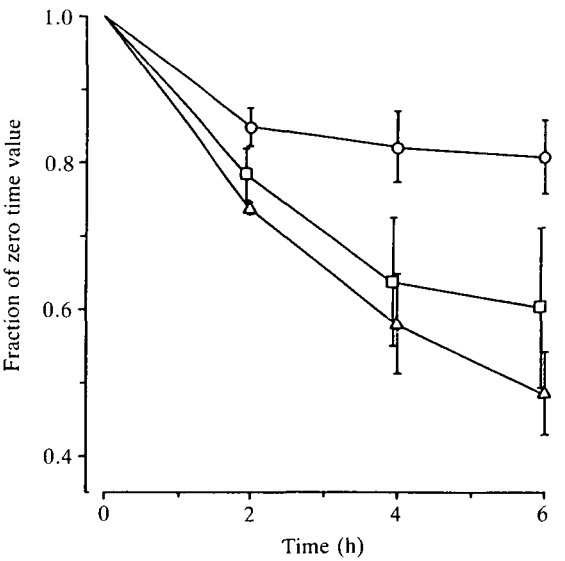

Fig. 2. Effect of metabolic inhibitors on red cell NTP. The influence of 500 μmol l^{-1} iodoacetate (□) and 100 μmol l^{-1} sodium cyanide (△) on red cell NTP is expressed relative to initial cellular content; ○, control. Values are mean±S.D. for iodoacetate (N=3), cyanide (N=3) and control (N=5). Relative to control values, the hematocrits at 6 h were 99±4 % (N=3) and 102±3 % (N=3) of the zero time value for iodoacetate and cyanide, respectively. Cells were pooled from 5–15 individuals from each of N different litters. The litters used in this *in vitro* study were 1–7 days old. A repeated-measures analysis of variance (SAS Institute, 1985) indicated that there is a significant (P=0.001) treatment times time linear interaction. This denotes that the slopes differ significantly between the control and the two treatments.

mals), adult values tend to be reached by 2–20 days after birth (Bard and Shapiro, 1979; Mueggler *et al.* 1980). As shown in Fig. 1, red cell NTP levels in the garter snake neonate rose quickly after birth and were not significantly different from adult male values at 6 h after birth. Furthermore, the P_{50} of red cells from 6-h neonates was not different from the value for the adult male. Therefore, red cell concentrations of ATP, the primary allosteric modifier of garter snake hemoglobin (Berner and Ingermann, 1988), as well as red cell oxygen-affinity apparently change very quickly after birth.

Incubation of neonatal red cells with sodium cyanide, an inhibitor of oxidative phosphorylation, and iodoacetate, an inhibitor of glycolysis, effectively reduced red cell NTP levels after several hours. That cyanide was particularly effective suggests that red cell NTP levels can be influenced by oxidative phosphorylation and oxygen availability. These results are consistent with those of Wood and Johansen (1972) and Greaney and Powers (1977, 1978) which suggested that low oxygen availability limits the ability of the nucleated fish red cells to generate ATP by oxidative phosphorylation.

If the fetus experiences hypoxia during parturition, we might expect red cell NTP concentrations to be lower just after birth than before. As illustrated in Fig. 1, there is no evidence for such a drop. However, based on the rate of NTP decrease in our *in vitro* experiments (Fig. 2), it seems unlikely that parturition involves a sufficiently long exposure to severe hypoxia or anoxia for the fetus to respond with a measurable change in red cell NTP concentration.

Our data are therefore consistent with the hypothesis that, for *T. elegans*, red cell ATP concentrations are controlled by oxygen availability and that these levels rise quickly after birth due to the onset of air-breathing. The concomitant rapid decrease in oxygen affinity at birth and the finding that ATP decreases the oxygen affinity of purified *T. elegans* hemoglobin *in vitro* (Berner and Ingermann, 1988) suggest that increased oxygen availability promotes the decrease in blood oxygen-affinity at birth. This decrease is likely to enhance the ability of the blood to deliver oxygen to the tissues. Therefore, the strategy of controlling blood affinity by oxygen availability *via* red cell ATP may be advantageous in hastening the ability of the neonate to function independently in its new environment.

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