Hypoxia, Hormones, and Red Blood Cell Function in Chick Embryos

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The red blood cell function of avian embryos is regulated by cAMP. Adenosine A₂A and β-adrenergic receptor activation during hypoxic conditions cause changes in the hemoglobin oxygen affinity and CO₂ transport. Furthermore, experimental evidence suggests a general involvement of cAMP in terminal differentiation of avian erythroblasts.

The chick embryo is an excellent model in which to study many aspects of embryonic red blood cell (RBC) development and function. Physiological parameters that govern blood gas transport, i.e., blood PO₂, PCO₂, and pH, have been established for all phases of development, and the developmental stages are easily accessible. Therefore, the properties of embryonic RBCs of all stages can be related to the prevailing physiological conditions, an advantage of the study of the avian over mammalian embryonic and fetal development. In Fig. 1A, the principal characteristics of the chick gas transport system are illustrated. Oxygen diffuses across the shell and the attached membranes into the capillary bed of the chorioallantois, which serves as the “lung” of the embryo and which replaces the yolk sac as the gas exchange organ at the end of the first week of development. As the embryo grows, the restrictions imposed by the fixed conductance of the shell and the limited diffusion capacity of the chorioallantois result in increasing diffusion gradients for O₂ and CO₂. As a result, progressive hypoxia and hypercapnia are normal features of later stages of embryonic development. To this set of ever-changing conditions, the embryonic RBC has to adapt its O₂ and CO₂ transport function. In contrast to RBCs of adults, embryonic RBCs are released into the circulation as erythroblasts, and therefore they act as gas transporters and complete terminal steps of erythroid differentiation. The basic processes of embryonic erythropoiesis are well established, but the functional characterization of the embryonic erythrocytes is still incomplete. One important question is to what extent the immature character of embryonic RBCs creates specific functional properties and regulatory mechanisms not observed in adult RBCs.

In the chick embryo, primitive RBCs produced in the yolk sac represent the cell population of the first week of embryonic development (Fig. 1B). At day 6, the first definitive erythroblasts appear in the circulation, which by day 8 constitute the majority of the circulating cells. While the RBC population expands rapidly (until day 17), the major portion of circulating RBCs is in the polychromatophilic/orthochromatich stage. Thus, for a period of >10 days, the chick embryo allows easy access to immature RBCs at different stages of differentiation.

This short review will detail 1) to what extent gas transport function of chick embryonic RBCs is regulated by hormonal effectors stimulating the cAMP pathway (norepinephrine (NE) and adenosine); 2) the development characteristics of the cellular cAMP signaling system; and 3) cAMP-dependent effects that point to a general role of cAMP in the penultimate steps of erythroid differentiation.

Adjustment of embryonic RBC function to developmental hypoxia: role of adenosine and NE

It is well established that embryonic RBCs of the chick and other avian embryos show rapid and extensive changes of their gas transport properties in later stages of development, which represents an adaptation to progressive hypoxia and hypercapnia. The hemoglobin oxygen affinity increases substantially (Fig. 1C; Ref. 13), allowing continuous adjustment to arterial hypoxia. In addition, the induction of erythroid carbonic anhydrase II (CAII) improves the CO₂ transport properties (Fig. 2A; Ref. 3) to oppose the negative effects of hypercapnia on hemoglobin oxygen binding (4).

As in other vertebrates, the oxygen affinity of avian embryonic hemoglobins is predominantly regulated by organic phosphates (Fig. 1C). The decline of the RBC nucleotides (ATP, UTP, CTP; Ref. 8) is largely responsible for the rapid increase of the oxygen affinity in late development. Whereas the RBC nucleotide concentration falls, the concentration of 2,3-bisphosphoglycerate (2,3BPG; a weak regulator of oxygen affinity in the chick embryo) increases transiently. Although these events have been extensively documented at the phenomenological level in the chick and other avian embryos, it is only recently that the hormonal control mechanisms responsible for this functional adaptation have been investigated in greater depth.

The search for signals regulating embryonic RBC function was initiated by the observation that moderate experimental hypoxia applied to a young chick embryo causes the same concerted changes in RBC function as in late development, that is an increase of hemoglobin oxygen affinity due to replacement of ATP by 2,3BPG and the activation of CAII synthesis (3). Furthermore, it was observed that embryonic plasma contains a hormonal factor(s) that induces the same changes during in vitro incubations of embryonic RBC preparations (14). These results led to the conclusion that the progressive hypoxia characteristic of late developmental stages is the primary drive for the adjustment of the hemoglobin oxygen affinity and CAII activity and that the hypoxic effect must...
be mediated via hormonal effectors. Further experiments identified NE and adenosine as hormonal effectors. Primitive and definitive erythrocytes of the chick embryo express β₁-like adrenergic and A_{2A} adenosine receptors (Fig. 3; Refs. 2, 7, and 10). Both are functionally coupled via G proteins to adenyl cyclase. Stimulation of adenyl cyclase with adrenergic agonists or adenosine and the resultant net increase of cAMP have in vitro the same effect on the embryonic RBCs as a hypoxic incubation of the intact embryo (Fig. 4; Refs. 7 and 10). Furthermore, hypoxia is the physiological stimulus for the release of large amounts of NE into the chick embryonic circulation. During normal development, plasma NE increases significantly at day 13 (Fig. 2A; Ref. 7), i.e., when the embryos become progressively hypoxic. Short-term hypoxia applied to younger embryos causes a fast increase in plasma NE (7). Since the release of NE is observed before the functional sympathetic innervation is established (11, 12), there will presumably be a direct release by hypoxia from the adrenal medulla, as has been described for mammalian fetuses. Together, these results lead to the conclusion that oxygen-dependent regulation of RBC function in late development relies largely on stimulation of cAMP signaling via NE. Less-direct evidence points to a role for adenosine in the regulation of normal RBC function. Although many cell types can release adenosine in response to hypoxia, adenosine cannot be directly measured in the embryonic blood due to the rapid degradation by RBC adenosine deaminase. Nevertheless, in day 6–7 embryos, hypoxia stimulates 2,3BPG and CAII synthesis by RBCs independently of catecholamines, pointing to a possible involvement of adenosine at this stage (7, 14). In addition, in day 13–14 chick embryos, in vivo blockade of adenosine receptors with antagonists attenuates the exchange of ATP by 2,3BPG (6). Finally, the combined blockade of β-adrenergic and adenosine receptors resulted in significant embryonic mortality, which underlines the general physiological importance of adenosine and NE at this stage. These results suggest that catecholamines and adenosine are not only involved in the control of cardiovascular development but are also critical for the adaptive regulation of embryonic blood gas transport properties.

The hormonal regulation of RBC function enables a flexible adjustment of the RBC gas transport properties by the actual PO₂. This fact may represent a prerequisite for the ability of birds to breed in regions above 4,000 m, because in embryos of birds adapted to high altitude (white-tailed ptarmigans, Lagopus leucurus), the RBCs are able to switch their organic phosphate pattern depending on the altitude of incubation (6).

**Developmental profile of cAMP signaling in embryonic RBCs**

To assess the putative importance of cAMP signaling throughout development, the response of the adenyl cyclase system was screened from early to late stages of embryonic development. During all stages, RBC adenyl cyclase is functionally coupled to β-adrenergic and adenosine A_{2A} receptors (Fig. 3; Ref. 2), although the intensity of the resulting cAMP response changes. Although primitive and early-to-midterm
definitive RBCs are highly responsive toward receptor agonists, RBCs from embryos older than day 15 become gradually less sensitive. The data suggest that the last steps of terminal differentiation to the mature erythrocyte are accompanied by extensive downregulation of the cAMP signaling pathway.

In general, there has been a dearth of studies analyzing cAMP signaling in immature RBCs, and for the most part the reported data have shown lower cAMP levels after receptor stimulation. However, whether this fact points particularly to a role of cAMP in embryonic RBCs or whether the models for the study of immature adult erythroid cells have been inadequate is still an open question.

In addition to the activation of adenylyl cyclase, the cAMP signaling strength is dependent on specific phosphodiesterases (PDE). Studies with embryonic RBCs suggest that PDE3, which is regulated by cGMP, plays a decisive role in the magnitude of the cAMP response (2). Under physiological conditions, PDE3 is apparently inhibited by cGMP, which is generated by nitric oxide (NO)-dependent guanylyl cyclase. Although there is no evidence for NO synthase (NOS) activity in embryonic RBCs, embryonic hemoglobin and glutathione can serve as erythroid NO storage reservoirs/donors via reversible binding of endothelial-derived NO. NOS activity has been demonstrated in endothelia of the chick embryonic chorioallantois (15). The dependence on PDE3 inhibition for a long-lasting cAMP signal creates a link between NO, cGMP, and cAMP signaling pathways. Thus the RBC cAMP system is embedded into a complex network of stimulatory/inhibitory pathways that subjects embryonic RBCs to signals from other embryonic tissues. This feedback may be necessary to adjust RBC function to changing developmental/environmental signals. Actually, the list of potential signals between embryo and RBCs can be expanded by cAMP itself, since it is released by embryonic chick RBCs and several recent studies give evidence that extracellular cAMP may act as a hormone in tissues of higher vertebrates (1).

cAMP-dependent control of CAII

Avian RBCs express the CAII isozyme. During chicken embryonic development, the CAII activity increases at day 14–15, which is time-coordinated to the increase in blood PCO₂. On the functional level, the increase of the CAII activity helps to minimize the negative effects of hypercapnia on blood pH and hemoglobin oxygen binding (4). Studies of the expression of CAII mRNA in circulating RBCs during development show a large increase in expression from day 13 onwards, with a peak at day 15 (Fig. 2A). At later stages, the expression declines in parallel with the ongoing shutdown of the overall transcriptional activity. The CAII transcription is activated by cAMP. In RBC preparations of day 11, the initially low mRNA level of CAII is increased in the presence of NE or adenosine receptor agonists followed by increased protein synthesis and CAII activity (Fig. 4). In addition, hypoxic incubation of day 10 embryos that increases the plasma NE level also stimulates erythroid CAII mRNA and protein synthesis (5).

During normal chick embryonic development, hypoxia and hypercapnia develop at the same time. Therefore, a regulation of CAII synthesis by hypoxia results in an adequate response toward developmental changes of the respiratory condition. The variation of blood PO₂ and PCO₂ that arises from differences in diffusive properties of eggshell and membranes directs the time course of the induction of erythroid CAII activity in the individual embryo. This adaptive control of CAII synthesis is reflected in large differences of the erythroid CAII activity of individual embryos late in development.

In vivo, an induction of CAII synthesis by hypoxia cannot be observed before day 7, although in vitro, day 5 RBCs respond to NE or adenosine receptor agonists with upregulation of the CAII mRNA (3, 5). The failure of early embryonic RBCs to respond to systemic hypoxia with persistent activation of CAII is presumably due to the fact that in early development the embryo relies largely on catecholamines provided by the egg yolk (12). However, this fact does not exclude that NE (and adenosine) play a role independent from the PO₂ in the differentiation of primitive RBCs.

Effect of cAMP on pyrimidine nucleotide metabolism

Stimulation of embryonic RBC by cAMP causes a pleio-
tropic response, because several nonglobin proteins are synthesized. Until now, only a few proteins besides CAII have been identified. Of particular interest is the finding that the embryonic RBC pyrimidine 5'-nucleotidase (PyN), a key enzyme of pyrimidine nucleotide degradation in RBCs, is induced by cAMP (8). In humans, a lack of the RBC enzyme causes a specific type of hemolytic anemia. Characteristically, RNA and ribosomes are retained in the circulating RBCs, suggesting that the effective degradation of cellular RNA during terminal erythroid differentiation is dependent on the presence of PyN. In the chick embryo, circulating RBCs lose their cellular RNA, leading to release of large amounts of mononucleotides. In early-to-midterm embryonic RBCs, the mononucleotides are presumably a major source for synthesis of nucleotide triphosphates. Besides ATP, the cells accumulate substantial amounts of UTP (and CTP; Fig. 1C; Ref. 8), both of which complement the action of ATP as the allosteric effector of hemoglobin. The time course for UTP accumulation is related to the course of PyN activity (Fig. 2B; Ref. 8). The PyN activity is high at early stages (day 4) but within 48 h declines to low values, which stay constant until days 13–14. The accumulation of UTP occurs until day 10 during the period of continuous RNA degradation and low PyN activity. Around days 13–14 the PyN activity transiently increases, with peak activity at day 15. The UTP/CTP levels decline at the same time. The increase in PyN activity is cAMP dependent (Fig. 4) and relies on transcriptional activation, and inhibition of transcription leads to downregulation of the enzyme (8). The cAMP-dependent induction results in enhanced release of uridine from the cells, indicating that the hydrolysis of monophosphates is the limiting factor for removal of pyrimidines from the RBCs. Together, the data suggest that the actual UTP/CTP content of embryonic RBCs depends on both the influx of UMP/CMP from RNA degradation and the activity of PyN, which diminishes the pyrimidine nucleotide pool. In addition, the breakdown of RBC UTP and CTP in late development makes RBCs into a significant source for release of pyrimidine nucleosides into the extracellular space. They can be used by other tissues, thereby avoiding the costly de novo synthesis at times of hypoxia.

Regulation of embryonic RBC ATP/2,3BPG

Although the effects of hypoxia on RBC ATP/2,3BPG are well known, and although there is ample evidence that ATP decrease and 2,3BPG increase are coupled, the underlying biochemical mechanism has not been unraveled for several reasons. On the one hand, the regulation of the purine nucleotide pool is complex because the pool size is adjusted by a combination of several enzymes (AMP deaminase, adenosine deaminase, adenosine kinase, and purine 5'-nucleotidase). On the other hand, the key enzymes involved in 2,3BPG synthesis are present and not regulated by hypoxia (3). It is therefore tempting to speculate that the activation of 2,3BPG synthesis depends on an increased glycolytic flux rather than de novo synthesis of specific enzymes. An important determinant for the glycolytic flow is set by the actual ATP concentration of the RBC: glycolysis is repressed in the presence of a high ATP concentration, and a fall of the free ATP concentration is required for its disinhibition. Indeed, published data show that 2,3BPG synthesis is never activated in RBC with ATP/hemoglobin ratios exceeding 2:1 (6). This suggests that the regulation of the purine nucleotide pool size is the critical control step of 2,3BPG synthesis.

Other cAMP-regulated processes related to erythroid differentiation

With immature RBCs of the chick embryo, the cAMP dependence of several characteristic processes during late RBC differentiation have been identified. In addition, several unknown proteins are induced in a cAMP-dependent manner. By differential cDNA library screening, we identified further cAMP-induced genes that are related to aspects of late erythroid differentiation but are not obviously linked to the gas transport. The first one found is the heat shock protein Hsp70.

FIGURE 3. cAMP response in chick RBCs during embryonic development. RBCs were stimulated for 5 min by β-adrenergic or adenosine A2-specific agonists. Adapted from Ref. 2.

FIGURE 4. Response of RBC preparations of day 11 chick embryos to β-adrenergic receptor activation. Shown are the CAII and PyN enzyme activity and 2,3BPG concentration after 16 h of incubation. Data are modified from Refs. 7 and 8. The mean values are expressed as % of the start level (100%) before incubation.
(5). It catalyzes folding and unfolding of proteins during the protein synthesis and degradation processes that occur in all cells. Embryonic RBCs of all stages already express Hsp70 on a substantial level, but in immature definitive RBCs the expression is upregulated in the presence of cAMP. The function of Hsp70 during differentiation of erythroid cells is obvious, because the reorganization and destruction of cellular organelles in late differentiation require extensive assistance in folding and unfolding proteins. In addition, Hsp70 might be needed for the increase of protein synthesis that is observed after cAMP stimulation (5).

A protein that is fast and strongly induced by cAMP in primitive (day 5) and definitive (day 11) RBCs is the transcription factor Fos (9). For transcriptional activations of specific genes, transcription factor binding to the promoter/enhancer regions must be activated. In the case of cAMP-dependent transcriptional regulation, it can happen either directly by protein kinase A-dependent phosphorylation of transcription factors (CREB or ATF-like proteins) or by new synthesis of transcription factors like Fos. The Fos protein binds to activator protein (AP)-1 sites as heterodimer with other transcriptional activators, like Jun. In the erythroid system of the chick embryo, the target genes of Fos are not yet determined, but AP-1 sites have been detected in a variety of erythroid gene loci.

Another interesting cAMP-induced gene of primitive and definitive RBC is the Tob protein (9). It belongs to the family of antiproliferative genes that are connected to several cellular signaling systems, like tyrosine kinase receptors, kinases, receptor serine/threonine kinases, and cyclin-dependent kinases. In all cases, Tob suppresses a mitogenic signal by binding to components of the signaling pathways. In the embryonic erythroid system, the connection to other signaling pathways remains to be investigated, but it opens up speculations that Tob (and therefore cAMP) might play a role in cell-cycle exit during earlier stages of erythropoiesis.

The last cAMP-induced erythroid protein investigated so far is the Ifr1 protein (9). Only a few functional studies have been carried out. In the mouse myoblast cell line C2C12, it seems to be a positive regulator of differentiation. By blocking the Ifr1 expression, a delay of the differentiated phenotype is attained by an unknown mechanism, with an impairment of myocyte-specific gene expression. In the erythroid system of the chick embryo, the putative role of Ifr1 as positive regulator of differentiation suggests a similar function.

Of course, the function of Fos, Tob, and Ifr1 in erythroid cells cannot be understood with this scarce knowledge. Nevertheless, the presence of Tob, Ifr1, and Fos in immature RBCs suggests that these proteins and their inducer cAMP have other, yet-undefined functions during final erythroid maturation.

Conclusions

The avian embryo is the first system in which oxygen-dependent hormonal regulation of RBC function during embryonic life has been established. The hormonal effectors are adenosine and NE, which stimulate adenyl cyclase. The cellular effects that are elicited by stimulation of the cAMP pathway are pleiotropic and are caused by extensive changes of transcriptional and translational activity. In consequence, the stimulation of the cAMP signaling pathway affects not only functions that have a direct bearing on RBC gas transport during embryonic development but also apparently affect the general process of terminal erythroid differentiation. Interestingly, both aspects are tightly coupled, as for example when the nucleotides derived from RNA degradation serve as allosteric effectors of hemoglobin to adjust the O₂ affinity.

Catecholamines have previously received great attention with respect to their role in the control of the cardiovascular system in embryonic development. The presence of catecholamines seems an indispensable requirement for normal development of the heart. The data obtained on immature RBCs underline the importance of catecholamines (and adenosine) during embryonic development because they are critically involved in the control of embryonic RBC function and terminal differentiation.

Perspectives

The general presence of β-adrenergic receptors on RBCs of adult higher and lower vertebrates has been established many decades, but only in a few cases has a significant functional effect been described, such as the stimulation of the Na⁺/H⁺ antiport in trout RBCs or volume increase via the Na⁺/K⁺-2Cl⁻ cotransporter in avian adult RBCs. A focus on more immature stages of RBC development could give further insights about the function of cAMP-dependent receptor signaling in terminal erythroid differentiation and control of RBC function.

References


