Highly efficient dissociation of oxygen from hemoglobin in Tibetan chicken embryos compared with lowland chicken embryos incubated in hypoxia

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ABSTRACT Oxygen is one of the critical determinants for normal embryonic and fetal development. In avian embryos, lack of oxygen will lead to high fetal mortality, heteroplasia, and cardiovascular dysfunction. Tibetan chicken is a breed native to Tibet that could survive and keep higher hatchability regardless of negative effects of hypoxia. Generally, adaptive animals in high altitudes are characterized by higher hemoglobin concentrations and oxygen affinity. In the present study, the capacity of oxygen supply in late chick embryo (including d 17, 19, and 21) was compared between Tibetan chicken and a lowland breed, Dwarf White chicken, by determining the hemoglobin concentrations and oxygen equilibrium curves in both hypoxic (13% O_2) and normoxic (21% O_2) conditions. The results showed that a higher level of hemoglobin concentration was induced by hypoxia in Tibetan chicken embryos, and the hemoglobin could perform with better cooperativity and deliver oxygen to tissues more easily. Further investigation revealed that the carbonic anhydrase II mRNA in red blood cells of Tibetan chicken was increasingly induced to a higher level in hypoxia than that of the lowland breed. These results suggested that the stronger capacity of oxygen dissociation was an important characteristic of Tibetan chicken embryo to survive in hypoxia and the upregulating mode of carbonic anhydrase II mRNA might assist this dissociation. Therefore, for avian at high altitudes, the efficient dissociation of oxygen might reveal another aspect associated with the hypoxia adaptability.

Key words: hypoxia, Tibetan chicken, hemoglobin, oxygen equilibrium curve, carbonic anhydrase II

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INTRODUCTION

When fertile eggs are hatched at high altitudes, the embryos are easily hurt by hypoxia. It has been reported that the hatchability of the sea-level breed was only 37% at a high altitude of 2,900 m compared with the normal hatchability of 90% at sea level (Wei and Wu, 2005). Tibetan chicken, an indigenous breed at high altitudes, is well known for its adaptability to survive in hypoxic conditions. When incubated at Lhasa with an altitude above 3,650 m, the hatchability of Tibetan chicken eggs could still be kept above 30%, but for sea-level breeds, it was less than 10% (Zhang et al., 2006). These reports indicate that Tibetan chicken could capture oxygen efficiently and hold tissular oxygen homeostasis with some special mechanisms in hypoxic condition. As the most important oxygen carrier found in vertebrates, hemoglobin has been the focus of hypoxia adaptation research for a long time because of its flexible concentration and oxygen affinity. So far, there are many examples demonstrating that avian species living at high altitudes are characterized by high oxygen affinity of hemoglobin (Perutz., 1983; Zhang et al., 1996; Liang et al., 2001). Gou (2005, 2007) has also reported a mutation of Tibetan chicken found in the α^{D} chain of hemoglobin that might enhance the oxygen affinity. But the explanation of high oxygen affinity for hypoxia adaptability may be just one reason; the other important one is to ensure efficient unloading of oxygen to deeper tissues, which is based on a flexible mechanism regulating the conformation of hemoglobin (Hiebl et al., 1987).

Generally, the interaction between hemoglobin and oxygen could be regulated by some small molecules, such as 2, 3-diphosphoglycerate, protons (H^+), NO, adenosine triphosphate, and modified hemoglobin (Kinoshita et al., 2007). Of these factors, the H^+ concentration in red blood cells (**RBC**) influenced by changing metabolic state is the most important factor that could easily change the shift of the dissociation curve and regulate oxygen affinity (Bellingham et al., 1971). At

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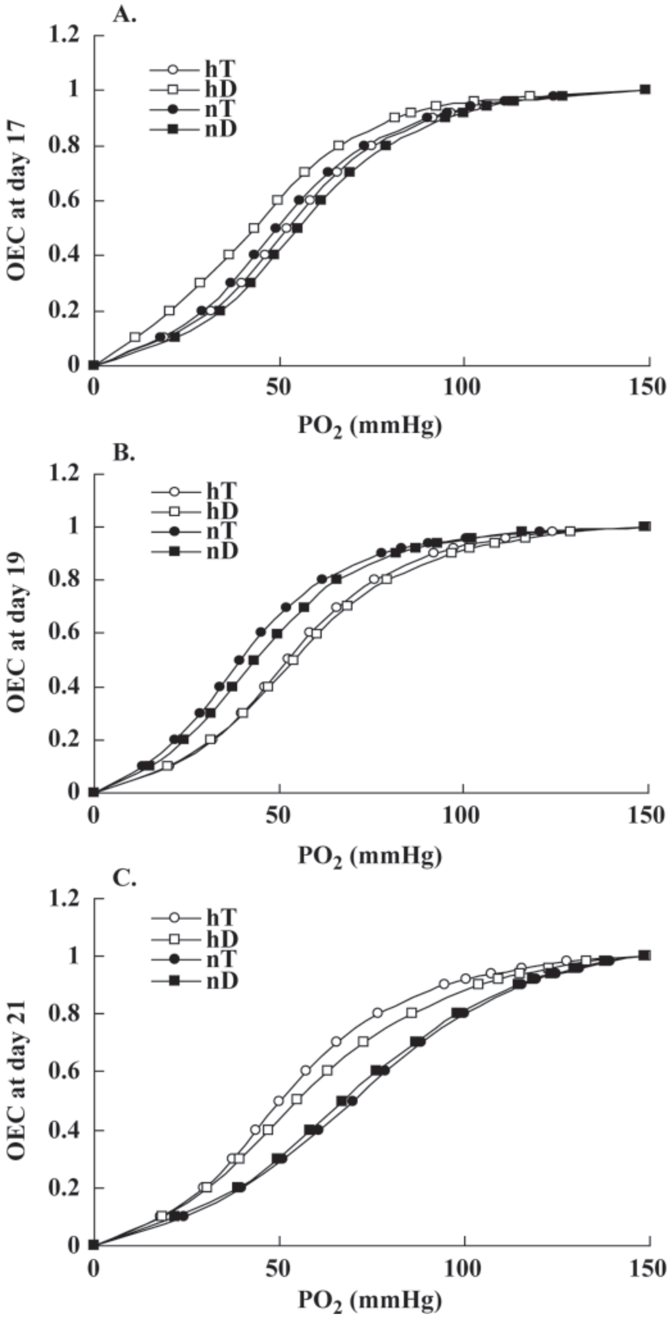

Figure 1. The erythrocyte oxygen equilibrium curve (OEC) of chick embryo in hypoxic and normoxic conditions. The curves show the aspects of oxygen affinity between Tibetan chicken (n = 5) and lowland chicken (n = 5) embryos at d 17 (A), d 19 (B), and d 21 (C). The data were obtained by using a Hemox analyzer (TCS Medical Products, Southampton, PA) in a standard condition (pH 7.4, 41°C) and then represented by graphs. hT = Tibetan chicken eggs incubated in hypoxia; nT = Tibetan chicken eggs incubated in normoxia; hD = lowland chicken eggs incubated in hypoxia; nD = lowland chicken eggs incubated in normoxia. PO_2 = partial pressure of O_2.

present, the reports on oxygen affinity of hemoglobin in poultries native to high altitudes are not available, especially in embryonic stages. In the following study, we compared the oxygen affinity of hemoglobin in Tibetan chicken embryos with that of the lowland chicken breed by the method of oxygen equilibrium curve (**OEC**; Kister and Wajcman., 2003) and found different shapes and positions of OEC in hypoxia, which might reflect an aspect of different adaptability. Previously, the work finished by Wei et al. (2007), describing the characteristics of higher venous carbon dioxide partial pressure and lower venous blood pH of Tibetan chicken embryos in hypoxia and suggesting some potential mechanism facilitating dissociation of oxygen from hemoglobin, has led us to pay attention to the carbonic anhydrase II (**CAII**) expression. Carbonic anhydrase II is a widespread zinc metalloenzyme from the carbonic anhydrase family that catalyzes $CO_2 + H^{+}OH^{-} \leftrightarrow HCO_3^{-} + H^{+}$ and plays a functional role in pH balance and dissipation of carbon dioxide in RBC (Geers and Gros, 2000). Therefore, the expression of RBC CAII mRNA of Tibetan chicken embryos was compared with those of lowland controls to understand how Tibetan chicken embryos could ensure oxygen supply and develop successfully in hypoxic condition.

MATERIALS AND METHODS

Materials

Fertile eggs of the Tibetan chicken and lowland chicken were collected from the Experimental Chicken Farm of the China Agriculture University. The fertile eggs were randomly divided into 2 groups: one group served as a control and was incubated in a normal condition (21% O_2) with 37.8°C and 60% RH for 21 d, and the other group was incubated by supplying with a gas mixture containing 13% O_2 and 87% N_2, simulating the oxygen partial pressure in the area of Lhasa approximate to an altitude of 4,000 m.

Measurement of OEC

The OEC was measured by using a Hemox analyzer (TCS Medical Products, Southampton, PA) in a standard condition (pH 7.4, 41°C). Blood samples were taken by cardiac puncture from 17-, 19-, and 21-d-old chick embryos with heparinized syringes and transferred to heparinized tubes kept at 4°C before use. All of the samples were assayed less than 8 h after cardiac puncture. After collection, the blood was centrifuged for 5 min at 350 × g at 4°C to remove the plasma and buffy coat. The packed erythrocytes were washed 3 times in cold 50 mM bis-Tris isotonic buffer, pH 7.4. An aliquot of the packed cells (60 μL) was suspended in 4 mL of the working buffer solution in the Hemox cuvet and was then prepared for measurement. The blood samples of Tibetan chicken embryos (n = 5) and lowland chicken embryos (n = 5) were measured at the same time in both hypoxic and normoxic incubation. The partial pressure of O_2 at half-saturation of hemoglobin ($\mathbf{P_{50}}$) and the Hill coefficient, reflecting the cooperative properties of hemoglobin ($\mathbf{n_{50}}$), were obtained as results of OEC measurement for affinity analysis.

Hemoglobin Concentration and Embryo Relative Weight

Hemoglobin concentration (g/100 mL) was measured following the method of cyanmethemoglobin (Tazawa et al., 1988). The eggs were weighed first, then eggshells were opened at the air cell and the embryos stripped of yolk were weighed. The egg weight was used as normalization for the embryo weight.

RNA Isolation and Analysis

Total RNA was extracted from erythroid cells using Trizol reagent (Invitrogen, Carlsbad, CA) and then purified with DNase I (RNase-free). The mRNA concentration was quantified for further analysis. The primers for the probe were designed using oligo 6.0 version according to the CAII gene (GenBank accession no. NW_001471651) sequence, and 18S rRNA was employed as a housekeeping gene for normalization. About 20 μg of total RNA was loaded onto 1.5% agarose gel for Northern blot following the standard protocol and then hybridized with [α-^{32}P] deoxycytidine triphosphate-labeled cDNA probes. The membranes were exposed to PhosphorImaging screens (Molecular Dynamics, Sunnyvale, CA) for 48 h and then signals were quantitated with ImageQuant software (Amersham Pharmacia Biosciences, Piscataway, NJ).

Statistical Analysis

Data analysis was performed using the GLM procedure of SAS v9.0 (SAS Institute Inc., Cary, NC). Differences between means were considered to be significant at the $P < 0.05$ level.

RESULTS

Oxygen Affinity

Tibetan chicken is well known for its stronger adaptability against hypoxia (Zhang et al., 2005, 2006; Bao et al., 2007). In this study, the result of OEC measurement showed that the curves were all induced to shift leftward in both breeds at d 17 and 21 but were induced to shift rightward surprisingly at d 19 in hypoxia, indicating no constantly enhanced affinity in embryonic stages (Figure 1). When the affinity was compared between breeds, it was surprising that higher affinity was even observed in lowland chicken embryos at d 17, and the mean P_{50} value was 43.29 ± 1.04 mmHg, far lower than 52.21 ± 1.68 mmHg in Tibetan chicken embryos ($P < 0.05$), which paralleled the value (49.42 ± 2.07 mmHg) in normoxia. At d 19 and 21, higher oxygen affinity of Tibetan chicken was shown in hypoxic condition but seemed not to be more excellent (only statistically different at d 21). At d 19, although lower mean P_{50} value in Tibetan chicken embryos was observed, there was no significance level ($P > 0.05$) in the mean P_{50} values between Tibetan chicken embryos (52.42 ± 0.82 mmHg) and lowland chicken embryos (55.84 ± 2.56 mmHg). At d 21, the mean P_{50} value in Tibetan chicken embryos (50.11 ± 1.26 mmHg) was significantly lower than that of lowland chicken embryos (54.68 ± 1.31 mmHg) ($P < 0.05$). In normal incubation, no significant difference of affinity between breeds was observed ($P > 0.05$). Although oxygen affinity of hemoglobin in Tibetan chicken embryos was not found to be more prominent in hypoxia, it seemed that the hemoglobin showed better tetramer cooperativity. In hypoxia, the mean n_{50} values of hemoglobin in both breeds were observed to be changed obviously. Compared with that of the lowland breed, the mean n_{50} value of hemoglobin in Tibetan chicken embryos was induced to be significantly higher ($P < 0.05$) at d 17 and 19 and still to be kept at a higher level but not at a significant level ($P > 0.05$) at d 21. In normal incubation, no difference of n_{50} values of both breeds was found ($P > 0.05$; Table 1).

Hemoglobin Concentration

No significant difference in hemoglobin concentration in normal incubation was observed between Tibetan chicken embryos and lowland chicken embryos at d 17, 19, and 21. In hypoxic incubation, hemoglobin concentration was upregulated by hypoxia, and hemoglobin concentration in Tibetan chicken embryos was significantly higher than that of lowland chicken embryos at d 17 and 19 ($P < 0.05$; Table 2).

Table 1. P_{50} and n_{50} values in Tibetan chicken and lowland chicken under different conditions[1,2]

		P_{50} value (mmHg)			n_{50} value		
Condition	Breed	Day 17	Day 19	Day 21	Day 17	Day 19	Day 21
Normoxia (21%)	Tibetan chicken (n = 5)	49.42 ± 2.07^a	41.00 ± 2.41^a	69.77 ± 1.89^a	3.00 ± 0.10^a	2.79 ± 0.10^a	2.95 ± 0.14^a
	Lowland chicken (n = 5)	53.38 ± 2.05^a	43.39 ± 2.12^a	67.27 ± 1.38^a	3.19 ± 0.22^a	2.60 ± 0.15^a	2.93 ± 0.03^a
Hypoxia (13%)	Tibetan chicken (n = 5)	52.21 ± 1.68^a	52.42 ± 0.82^b	50.11 ± 1.26^b	3.23 ± 0.12^a	3.29 ± 0.03^b	2.93 ± 0.03^a
	Lowland chicken (n = 5)	43.29 ± 1.04^b	55.84 ± 2.56^b	54.68 ± 1.31^c	2.29 ± 0.10^b	3.06 ± 0.07^c	2.69 ± 0.13^a

[a–c]Means within the same row with no common superscript differ ($P < 0.05$).

[1]Values represent the mean ± SE.

[2]P_{50} = partial pressure of O_2 at half-saturation of hemoglobin; n_{50} = Hill coefficient, reflecting the cooperative properties of hemoglobin.

Table 2. The hemoglobin concentration (g/100 mL) of Tibetan chicken and lowland chicken embryos incubated in hypoxic (13%) and normoxic (21%) conditions[1,2]

Day	hT (n = 10)	nT (n = 10)	hD (n = 10)	nD (n = 10)
17	12.67 ± 0.61^{a}	9.16 ± 0.32^{b}	10.82 ± 0.29^{c}	9.11 ± 0.32^{b}
19	14.07 ± 0.29^{a}	10.40 ± 0.50^{b}	12.04 ± 0.57^{c}	10.22 ± 0.57^{b}
21	12.14 ± 0.74^{a}	11.34 ± 0.92^{ab}	9.03 ± 0.49^{b}	11.31 ± 0.63^{ab}

[a–c]Means within the same row with no common superscript differ ($P < 0.05$).

[1]Values represent the mean ± SE.

[2]hT = Tibetan chicken eggs incubated in hypoxia; nT = Tibetan chicken eggs incubated in normoxia; hD = lowland chicken eggs incubated in hypoxia; nD = lowland chicken eggs incubated in normoxia.

Relative Embryo Weight

In the normal condition, lowland chicken showed a higher embryo weight than that of Tibetan chicken. But when incubated in hypoxia, it was observed that the embryo weight of lowland chicken decreased more than that of Tibetan chicken, especially in the late stages from d 17 to 21, showing no significant difference in weight compared with Tibetan chicken embryos (Figure 2A). To accurately compare the growing state between the 2 breeds, egg weight was used to normalize the embryo weight. The result showed that the value of embryo:egg rate in Tibetan chicken embryo was significantly higher than that of the lowland breed in hypoxic condition ($P < 0.01$), but there was no difference observed in the normal condition (Figure 2B).

CAII mRNA Expression

To investigate the Bohr effect on hemoglobin, we analyzed CAII mRNA expression in RBC of late embryos at d 17, 19, and 21 in hypoxic and normoxic incubation (Figure 3A). Northern blot showed that CAII mRNA level could be significantly upregulated and even expressed earlier at d 13 in hypoxia. The level of CAII mRNA in Tibetan chicken in hypoxic condition was found to be significantly higher than that of lowland chicken (Figure 3B), and no different expression was found in normoxic incubation.

DISCUSSION

Hemoglobin concentration and oxygen affinity are the most important factors, responsible for ensuring oxygen content in blood against hypoxic condition. Previous studies demonstrated that the hemoglobin concentration in avain embryos could be greatly induced at high altitudes (Carey et al., 1993), and the intensified oxygen affinity was constantly considered to be a key factor for hypoxia adaptability (Lapennas and Reeves, 1983). Baumann et al. (1983, 1987) have reported that the P_{50} curve in chick embryos was shifted to the left in hypoxia during early or middle stages and the shift of curve enhanced oxygen affinity from d 6 to 14. In our study, a higher level of hemoglobin could be observed in both breeds in hypoxia, consistent with previous reports. However, the oxygen affinity of embryos was not observed to be enhanced all the time in hypoxia, although the change of hemoglobin P_{50} value showed significantly different aspects in hypoxic and normoxic conditions. Especially at d 19, the mean P_{50} values in both Tibetan chicken embryos and the lowland breed increased significantly compared with those in normoxia, indicating that the oxygen affinity was reduced in hypoxic condition. Does this mean abnormality? In general, chick embryos have relatively af-

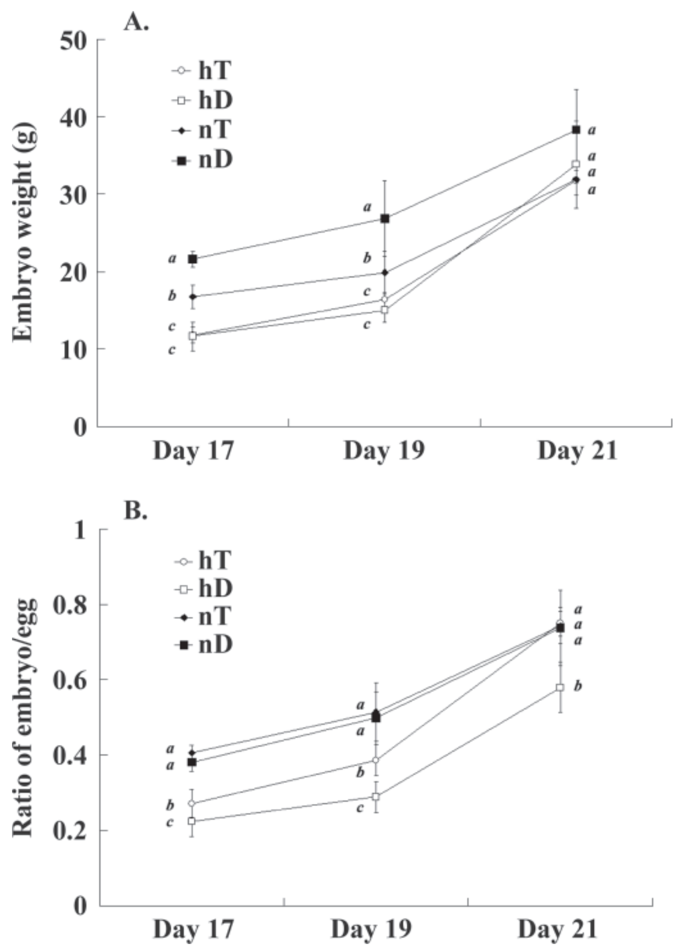

Figure 2. Comparison of growth between Tibetan chicken and lowland chicken embryos at d 17, 19, and 21. (A) The curves of embryo weight are shown in hypoxic and normoxic incubations, respectively. (B) Embryo weight normalized to egg weight is used as a standard to depict the growth of chick embryos in hypoxic and normoxic incubation, respectively. Results statistically different are indicated with a letter, and values sharing no common letter differ ($P < 0.05$). hT = Tibetan chicken eggs incubated in hypoxia; nT = Tibetan chicken eggs incubated in normoxia; hD = lowland chicken eggs incubated in hypoxia; nD = lowland chicken eggs incubated in normoxia.

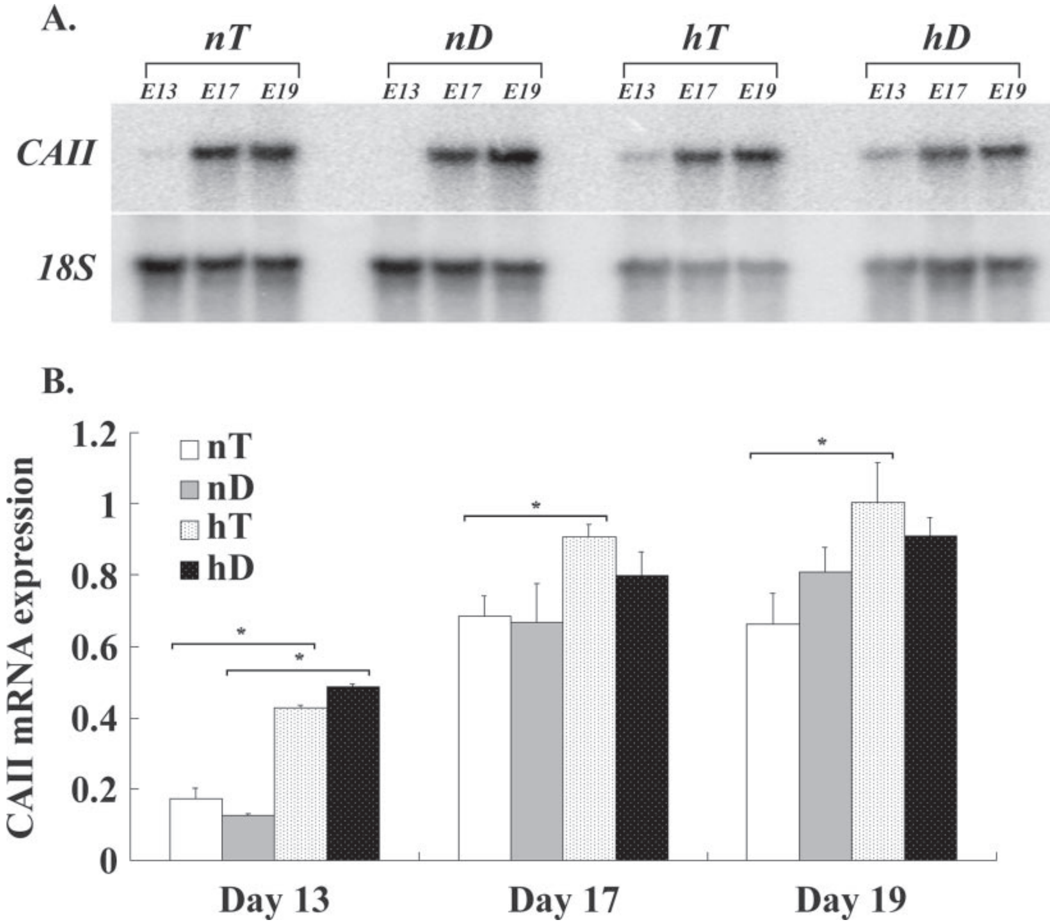

Figure 3. Expression of carbonic anhydrase II (CAII) mRNA in different chicken breeds under hypoxic (13%) and normoxic (21%) conditions, respectively. (A) The level of CAII mRNA is measured by Northern analysis, and 18S rRNA is used as control for normalization. (B) The level of mRNA expression is shown by comparison of signal intensity of lanes, and each bar represents the mean ± SE for each group with the erythroid pool RNA (n = 5) of embryos. Results statistically different ($P < 0.05$) are indicated with an asterisk (*). hT = Tibetan chicken eggs incubated in hypoxia; nT = Tibetan chicken eggs incubated in normoxia; hD = lowland chicken eggs incubated in hypoxia; nD = lowland chicken eggs incubated in normoxia. E13, E17, E19 = embryonic d 13, 17, and 19, respectively.

fluent oxygen in the early stages compared with the late stages (Freeman and Misson, 1970; Tazawa and Mochizuki, 1977; Meuer and Baumann, 1988) because the embryo consumes more oxygen and grows faster in late stages, as observed in our results. Therefore, it was possible that a higher level of hemoglobin induced in hypoxia compensated for the loss of oxygen affinity and ensured oxygen intake. Therefore, we assumed this shift of curves at d 19 in hypoxia was normal and probably reflected the importance of oxygen dissociation during late chicken embryonic stages. When compared between the 2 breeds, the importance of oxygen dissociation seemed to be clearer. During all late stages (d 17, 19, and 21), the Tibetan chicken embryos in hypoxia did not show a better affinity for oxygen than lowland chicken embryos, and the mean P_{50} value was not even influenced by hypoxia at d 17. The mean n_{50} values in Tibetan chicken embryos were kept at a constant level, significantly higher than that of lowland chicken embryos at d 17 and 19. The Hill coefficient is generally used for measuring tetramer cooperativity, and higher values mean efficient deoxygenation or reoxygenation of hemoglobin. The differing values between the 2 breeds probably suggested that Tibetan chicken had a more flexible regulation in hemoglobin affinity. Wei et al. (2007) has reported that Tibetan chicken embryos could keep a lower pH in blood in hypoxia, indicating that the regulation would be more easily influenced by the Bohr effect. Therefore, based on the fact that higher oxygen content in blood was ensured by higher hemoglobin concentration in Tibetan chicken embryos, we hypothesized that efficient oxygen dissociation was a specific mode for Tibetan chicken embryos against hypoxia and the higher Bohr effect might ensure higher tissue oxygen pressure by facilitating oxygen dissociation from hemoglobins.

To investigate this hypothesis, we detected the level of CAII mRNA in RBC. Carbonic anhydrase II is a kind of zinc metalloenzyme that catalyzes the reversible reaction of carbon dioxide and water to bicarbonate and protons. In erythrocyte, CAII is responsible for improving the oxygen and carbon dioxide transport properties and regulating hemoglobin oxygen binding dependent on the Bohr effect (Baumann et al., 1986; Birchard and Black, 1986). Dragon and Baumann (2001) have previously reported that CAII mRNA transcription could be induced by hypoxia. In our study, we confirmed this by Northern blot and found a higher level of CAII mRNA in Tibetan chicken embryos, indicating that the upregulation of CAII transcription in hypoxia would enhance translation quantity. Therefore, we speculated that higher expression of CAII in Tibetan chicken embryos might play an important role in regulating cellular pH and facilitating more oxygen to dissociate from hemoglobin to tissues for metabolic consumption in hypoxic incubation. Handrich and Girard (1985) have reported that embryo weight and oxygen consumption were strongly correlated. In our study, Tibetan chicken embryos indeed showed a higher rate of relative weight in hypoxic incubation, indicating that more efficient oxygen was used for their embryo development. Therefore, we thought that the flexible regulation of hemoglobin in Tibetan chicken embryos by the Bohr effect was one aspect of hypoxia adaptability at a high altitude.

In addition, hypoxia-induced upregulation of CAII mRNA might be a potential factor associated with hypoxia adaptability. A previous study reported that CAII transcription was activated through a cyclic adenosine monophosphate-dependent signaling pathway induced by adenosine and norepinephrine (Dragon and Baumann, 2001), but hypoxia-inducible factor 1 should not be neglected because the hypoxia response element in the promoter of carbonic anhydrase IX, another carbonic anhydrase isozyme, had been confirmed (Kaluz et al., 2003). In our study, CAII induced by hypoxia in Tibetan chicken embryos was a necessary reinforcement for facilitating dissociation of oxygen to tissues and might also be one of the important factors in the hypoxia adaptability mechanism. Of course, further studies are still required.

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