Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part B 147 (2007) 147–155
CBP
www.elsevier.com/locate/cbpb

Hypoxic adaptations of hemoglobin in Tibetan chick embryo: High oxygen-affinity mutation and selective expression

Xiao Gou [a], Ning Li [b], Linsheng Lian [a], Dawei Yan [a], Hao Zhang [c], Zhehui Wei [c], Changxin Wu [c,*]

[a] College of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, China
[b] The National Opened Laboratory of Agrobiotechnology, China Agricultural University, Beijing 100094, China
[c] College of Animal Science and Technology, China Agricultural University, Beijing 100094, China

Received 9 September 2006; received in revised form 28 October 2006; accepted 23 November 2006
Available online 24 January 2007

Abstract

Tibetan chicks (*Gallus gallus*) survived with high hatchability (35.0%) and Recessive White Feather broilers (RWF) from low elevations survived rarely and with a low hatchability (3.0%) after simulated incubation under hypoxia of 13% O_2. The functional mutation of Met-32D (B13)-Leu of α^D globin chain was related with hypoxia based on allele distribution, homology model building and oxygen affinity assay. Whole embryos on days 3–8 and whole blood on days 9–18 were collected to investigate the stage expression profiles of all seven globins and HIF-1α by real-time PCR. Under hypoxia (12.0% O_2) on days 3–8, HbE was overexpressed, HbA was expressed earlier and HbP expression was restricted, which completely overturned the expression profile under normoxia. The amount of hemoglobin expression in Tibetan chicks was remarkablely higher than that of RWF. HIF-1α expression peaked early in both breeds, with. In conclusion, the special hypoxic expression profile on days 3–8 certainly is a common molecular mechanism of hypoxia tolerance in surviving Tibetan chick and RWF embryos; the mutation Met-32D(B13)-Leu and increasing hemoglobins are important mechanisms of hypoxia adaptation in Tibetan chick embryos, and we suggest that HIF-1α could be responsible for the hypoxic expression profile.

Keywords: Tibetan chick; Embryo; Hypoxia; Hemoglobin; Hatchability; HIF-1α

1. Introduction

Tibetan chick, as an indigenous chicken (*Gallus gallus*) bred at high altitudes, is well known for its high hatchability under conditions of reduced oxygen supply. The hatchability of low land breeds is approximately zero under hypoxia at simulated altitudes of above 4000 m (Visschedijk, 1980, 1985), while the natural hatchability of Tibetan chick can reach 70–80%. We devised an incubator that can automatically control oxygen concentration, to investigate the difference of hypoxia adaptation between Tibetan chicks and Recessive White Feather broilers (RWF).

The high oxygen affinity of mutated hemoglobin is an important molecular mechanism of hypoxia adaptation in high altitude species. Bar-headed geese (*Anser indicus*) live at an altitude of 4000–6000 m and migrate annually across the summit of Mt. Everest to the Bengal Gulf. The mutation Pro-119α(H2)-Ala, responsible for the high oxygen affinity of the bar-headed goose Hb, leaves a two-carbon gap and relaxes the tension in T (tense) structure because Pro is mutated to smaller Ala (Perutz, 1983; Weber et al., 1993; Zhang et al., 1996; Liang et al., 2001). Other birds such as Andean goose (*Chloephaga melanoptera*), tufted duck (*Aythya fuligula*), white-headed vulture (*Trigonoceps occipitalis*) and black vulture (*Aegypius monachus*) have similar hemoglobin molecular mechanisms (Jessen et al., 1991; Abbasi and Lutfullah, 2002; Lutfullah et al., 2005; Hiebl et al., 1989). However, there has been no description on oxygen affinity of Tibetan chick hemoglobins, thus we have sequenced all exons of hemoglobins of Tibetan chick and RWF, determined blood-oxygen affinity and predicted the function of hemoglobin mutation with reference to hypoxia adaptation.

Seven types of hemoglobins exist in chicks: HbA, HbD, HbP, HbM, HbE, HbL and HbH, which are composed of $\alpha_2^A\beta_2^A$, $\alpha_2^D\beta_2^A$, $\pi_2\rho_2$, $\alpha_2^D\varepsilon_2$, $\alpha_2^A\varepsilon_2$, $\alpha_2^D\beta_2^H$ and $\alpha_2^A\beta_2^H$, respectively. The

* Corresponding author. Tel.: +86 1062733683.
E-mail address: lingzi@cau.edu.cn (C. Wu).

1096-4959/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2006.11.031

primitive erythroid cells produce five globins ρ, ε, π, α^D and α^A, and the definitive erythroid cells, replacing the primitive erythroid cells at about day 5, mainly express adult globins β^A, β^H, α^A and α^D. The embryonic ρ globin is most actively expressed at the sixth day and the fetal β^H globin is expressed from day 17 to 22 (Brown and Ingram, 1974; Chapman et al., 1981, 1982a,b; Landes et al., 1982; Cirotto et al., 1987). Obviously, the types and amounts of chick embryo hemoglobins are gradually changed with increasing development age. However, it is unclear if the individual types of hemoglobins adaptatively change in chick embryo under hypoxic condition. Therefore, we have analyzed and determined mRNA hypoxic expression profile of all seven globins and HIF-1α (Hypoxia-inducible transcription factor-1α) (Takahashi et al., 2001; Etchevers, 2003) in Tibetan chick and RWF using real-time PCR to further reveal molecular mechanisms of hypoxia adaptation in Tibetan chick.

2. Materials and methods

2.1. Sources of samples and simulated incubation under hypoxia

Two groups of Tibetan chicks in incubation experiments were raised in the chick farm of China Agricultural University, one of which was from different altitudes in Tibet and another from a high altitude of 2600 m in Tibet. The fertilized eggs of RWF were gathered from the chick farm of China Agricultural University. The blood or tissue of adult individuals was randomly collected. For Tibetan chick, 28 individuals were from altitude of 3600 m in Tibet, 24 individuals from altitude of 3000 m in Yunnan province and 22 individuals were from altitude of 2600 m in Tibet. In addition, 50 individuals of Guangxisanhuang chick and 50 individuals of Beijingyou chickens were collected from the chick farm of China Agricultural University.

Hypoxia incubator was devised by refitting a small incubator to control O_2 concentration by changing N_2 concentration but keeping other incubation conditions normal with temperature of 37.8 °C, relative humidity of 70%, and turning eggs every 2 h. The oxygen concentrations of the two incubations were 13.0% and 12.0%. During hypoxia incubation the incubator was kept airtight to maintain the oxygen concentration. The death time was identified after opening the eggshell on day 21.

2.2. PCR amplification and sequencing

Based on chick α-like globin sequences (Genbank accession no. AY016020) and β-like globin sequences (Genbank accession no. L17432), 19 primer sets were designed to amplify the seven globin genes containing all 21 exons. These primer sequences are showed as follows and A, D, P, B, R, E and H represent the primers of α^A, α^D, π, β^A, ρ, ε and β^H globins, respectively.

A11, 5′GGCCAGCACAGCATATAAGG3′; A12, 5′ TAAGGCAGGGAGGGATAGGA3′.
A21, 5′CCGAGACCCTGGAAAGGTAG3′; A22, 5′ AAAAGGAGAGGATGAGCGAG3′.
A31, 5′CACCCCCTCGCTCATCCTCTCC3′; A32, 5′ CATCTCATTTGGCTGCTCGCTGTC3′.
B11, 5′ACAACCACACGCTACCCTCCAACC3′; B12, 5′ CTCTCCCACCTCCGACCCTGAAC3′.
B21, 5′CACATTGCGCATTTTGA3′; B22, 5′ CCGAGCTGCACAGGGACACA3′.
D11, 5′ACCGCCGCTCCCTGCTCTCA3′; D12, 5′ TGCCCCCTGCCCCAACC3′.
D21, 5′AGGGCAGGGCAGGGCAGCAGT3′; D22, 5′ AGGTCGAAGTGGGGGAAGTAGGTC3′.
D31, 5′CTGCGGGTCCTGGGGTCCTCA3′; D32, 5′ GCTGCAGCAATGGTGTCTTTA3′.
P11, 5′GGCCCGCTCTCCTCTTCCTC3′; P12, 5′ TTCATAGCACGGGTGTAACTCCAG3′.
P21, 5′TAATATAGGAAACGTGGTGTCAGT3′; P22, 5′ ATCCAGTCTCAGGTTAAAAATCAG3′.
P31, 5′ TAGAGGCCACAGGTCATTTAG3′; P32, 5′ AAGCCATTTATCTGTATTTCTCA3′.
R11, 5′GCCGCCTGTTAAGCTGGTCAC3′; R12, 5′ AGCGCTGCCTCCTAAAACTGC3′.
R21, 5′GTGTGCTTGTGTCCCCCGTCTC3′; R22, 5′ ACTCCCCTCCTGCTTCCCTCCTTC3′.
R31, 5′TGCTTACCCCATTGCTCCCCTTC3′; R32, 5′ CTGCCCCCACATGTCCCCTGA3′.
E11, 5′CACCCCGACCACCCCCTCCTG3′; E12, 5′ AGCACTGCCTCCTAAAACTG3′.
E21, 5′GTGTGCTTGTGTCCCCCATCC3′; E22, 5′ CAGCCTCACAATCCTTTCTCCTTC3′.
E31, 5′CCAAACCGGCACCCTAAGA3′; E32, 5′ CCCCATACATCCCCCAAAACAGAC3′.
H11, 5′TGGCCCCACTTCCTACTCATCG3′; H12, 5′ CGCCTGCTTGCATCCCTCCTC3′.
H21, 5′AGCCAGGGGAGGGGAGAAATGAAG3′; H22, 5′AGCCCAGCAGACCCCGAGGATG3′.

50 ng of total DNA of blood was used to PCR amplification in 50 μL of PCR mixture containing 10 pM each of primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 2U *Taq* DNA polymerase. PCR was performed under the following conditions: DNA denaturation at 95 °C for 5 min, then 35 cycles of denaturation at 94 °C for 1 min, annealing at 55–58 °C for 1 min, and extension at 72 °C for 1 min. The purified PCR products were directly sequenced using the ABI PRISM Dye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and were electrophoresed on an ABI 377 PRISM DNA sequencer (Applied Biosystems).

2.3. Gene quantification

Specimen was collected from chick embryos incubated under hypoxia of 12.0% O_2. The whole embryo on days 3–8 included allantochorion and whole blood. The blood on days 9–18 was obtained by puncturing through veins of allantochorion. The blood on days 9–18 was directly digested using Trizol reagents (Invitrogen) and the frozen whole embryo was totally