A Comparison of Mitochondrial Respiratory Function of Tibet Chicken and Silky Chicken Embryonic Brain

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ABSTRACT The Tibet chicken lives in high altitude and has adapted itself well to hypoxia. The Silky chicken is a lowland chicken from Jiangxi province of China. The objective of the present study was to investigate whether there were any differences in brain mitochondrial respiratory function between Tibet chicken and Silky chicken embryos incubated in a normoxic (21% oxygen concentration) or simulated hypoxic (13% O_2) hatchibator. Brain mitochondria of chicken embryos were prepared by differential centrifugation on d 16 of incubation. The respiratory control ratio (RCR) and the adenosine 5′-diphosphate:oxygen ratio (ADP/O) were determined polarographically. The complex I activity was measured with an ultraviolet spectrophotometer by following the oxidation of the reduced state of β-nicotinamide adenine dinucleotide. Under the normoxic incubation condition, there were no significant differences in the RCR, the ADP/O, and the activity of complex I between embryonic brain mitochondria of the 2 breeds. Under the hypoxic incubation condition, the ADP/O in brain mitochondria of embryos from the 2 breeds were identical. Also under hypoxic conditions the RCR in brain mitochondria of Tibet chicken embryos was higher ($P < 0.05$) than in Silky chicken embryos when brain mitochondria were provided with glutamate-malate, but no significant difference was found in the RCR with succinate as an energy substrate. The complex I activity of Silky chicken embryos was higher than that of Tibet chicken embryos when they were incubated in the hypoxic hatchibator ($P < 0.01$). In conclusion, the results show that under simulated hypoxic incubation conditions electron transport in brain mitochondria of Tibet chicken embryos was more tightly coupled than that of lowland chicken (Silky chicken) embryos with glutamate-malate as energy substrate, which was associated with the difference in the activity of complex I between embryonic brains of the 2 breeds. This work will provide reference for future studies on the association of mitochondrial respiratory function with the adaptation to hypoxia.

Key words: mitochondrial respiratory function, Tibet chicken, Silky chicken, embryonic brain, hypoxia

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INTRODUCTION

Mitochondrial oxidative phosphorylation accounts for about 90% of cellular oxygen uptake and provides more than 80% of the energy need for cellular life metabolism (Papa, 1996). The brain is the organ with the highest demand for aerobic adenosine triphosphate (**ATP**) production, consuming 20% of oxygen uptake with 2% of body mass at rest (Erecinska and Silver, 1989). Hypoxia is an aggressive physiological stressor inducing wide deleterious effects. It has been showed that exposure to hypoxia causes retarded development, increased mortality, prolonged incubation periods, and smaller hatchling masses of avian embryos (León-Velarde and Monge-C, 2004). Pulmonary hypertension syndrome in chickens is associated with subnormal levels of arterial blood oxygenation, and its incidence is directly related to the level of hypoxia (Odom et al., 2004). Mitochondrial morphological changes, such as considerable swelling and cristae degeneration, were found in rat and human mitochondria (Santos et al., 2002; Hoppeler et al., 2003; Schild et al., 2003) exposed to hypobaric hypoxia. Hypoxia increases oxidative stress (Magalhães et al., 2005) and enhances the production of reactive oxygen species (Duranteau et al., 1998), which may damage membrane lipids, affect mitochondrial enzyme activities, and consequently impair mitochondrial function (Paradies et al., 1999, 2001, 2002; Petrosillo et al., 2001; Magalhães et al., 2005).

In general, highland native animals have genetically adapted themselves to hypobaric hypoxia. Significant differences of mitochondrial volume density were found between highland and lowland native humans (Kayser et al., 1991, 1996), but very little was known about differences in mitochondrial respiratory function between highland and lowland native animals of the same species. The Tibet chicken lives in Qinghai-Tibet Plateau in the west of China and adapted itself well to hypoxia, which is mainly demonstrated by the observation that the native

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chicken has higher hatchability at high altitude than lowland chicken breeds (Zhang et al., 2005, 2006). The Silky chicken is a lowland chicken from Jiangxi province of China, with an altitude of 750 m. The hatchabilities of the Tibet chicken and Silky chicken were about 85% in a normoxic hatching condition (21% oxygen concentration). However, in a simulated hypoxic hatchibator (designed by our laboratory) with 13% oxygen concentration, the hatchabilities of Tibet chicken and Silky chicken were about 40 and 2%, respectively (data not shown). The objective of the present study was to investigate whether there was any difference in brain mitochondrial respiratory function between Tibet chicken and Silky chicken embryos incubated in a normoxic (21%) or simulated hypoxic (13%) hatchibator.

MATERIALS AND METHODS

Hens and Eggs

Silky chicken hens and Tibet chicken hens of the same age were raised in the Experimental Chicken Farm of China Agricultural University with the same management procedure. At the end of wk 40, they were fertilized by artificial insemination. A total of 300 Silky chicken and 160 Tibet chicken breeding eggs were collected within 3 d. Sixty Silky chicken and 52 Tibet chicken eggs were incubated in a normoxic hatchibator and 240 Silky chicken and 108 Tibet chicken eggs were incubated in a simulated hypoxic hatchibator with 13% oxygen concentration.

Sampling Procedure

On d 16 of incubation, eggshells were opened at the air-cell and living embryos were pulled out with a nipper, and brains were obtained and immediately put into 2-mL microcentrifuge tubes in an ice bath for isolation of mitochondria.

Mitochondrial Isolation

Brain mitochondria were prepared by differential centrifugation, as described by Clayton and Doda (2001) with a little modification. Briefly, brains were immediately excised and finely minced in ice-cold isolation medium (225 m*M* mannitol, 75 m*M* sucrose, 1 m*M* EDTA, 50 m*M* Tris-HCl, and 0.2% BSA, pH 7.4). The sample was then carefully homogenized in a Potter-Elvenhjem vessel with a Teflon pestle of 0.1-mm clearance. After homogenization, 3 volumes of isolation medium were added to the homogenate, which was then fractionated by centrifugation at 1,300 × *g* for 10 min. The supernatant was decanted into a new tube placed in ice. The pellet was gently resuspended in 2 volumes of isolation medium and centrifuged at 1,300 × *g* for 10 min; then the supernatant was decanted into the new tube and mixed with the first supernatant and the pellet was discarded. The total supernatant was centrifuged at 17,000 × *g* for 15 min. The supernatant was discarded, and the pellet was gently resuspended in isolation medium (0.5 mL/100 mg of initial tissue) and centrifuged at 17,000 × *g* for 10 min. The supernatant was discarded, and the final pellet, containing the mitochondrial fraction, was gently resuspended (2 μL/mg of initial tissue) in reaction buffer (225 m*M* mannitol, 75 m*M* sucrose, 1 m*M* EDTA, 10 m*M* KH_2PO_4, and 0.1% BSA, pH 7.4). All mitochondrial isolation procedures were performed at 0 to 4°C. Mitochondrial protein concentration was spectrophotometrically estimated with the Bradford method using bovine serum albumin as standard. The mitochondrial suspensions were used within 3.5 h after the isolation and were maintained on ice (0 to 4°C) throughout this period. There was no decline in mitochondrial respiratory function in mitochondria isolated at the beginning and end of the experiment within each group.

Determination of Mitochondrial Function

Mitochondrial respiratory function was measured polarographically with Strathkelvin 782 Oxygen System (Strathkelvin Instruments Ltd., Glasgow, UK) according to the protocols described by Zhou et al. (2003) and Beijing SBS Biotechnology Company, Beijing, China. The respiratory control ratio (**RCR**) and the adenosine 5′-diphosphate:oxyen ratio (**ADP/O**) are 2 main parameters of mitochondrial respiratory function. The RCR, an index of electron transport chain coupling, is calculated as state 3 divided by state 4 respiration rate. The ADP/O is the amount of ADP utilized per nanomole of oxygen consumed during state 3 respiration and is an index of the ability of mitochondria to carry out oxidative phosphorylation of ATP (Cawthon et al., 1999). Reactions were conducted in 1.5-mL closed thermostated (25°C) glass chambers equipped with magnetic stirring. Aliquots (0.1 mL, ~1.0 mg of protein) of the brain mitochondria were added to the reaction vessel containing 0.4 mL of the reaction buffer. The substrates tested in this study were glutamate-malate (2:2 m*M*) and succinate (4 m*M*), which donate electrons to the respiratory chain at complex I and complex II, respectively. After 2 min equilibration, mitochondrial respiration was initiated by adding glutamate-malate or succinate. State 3 respiration was started after adding ADP (200 μ*M*, final concentration), followed by state 4 respiration when ADP was consumed.

Complex I Activity

Complex I [the reduced state of β-nicotinamide adenine dinucleotide (**NADH**) ubiquinone:oxidoreductase] activity was assessed by ultraviolet spectrophotometry. Briefly, complex I activity was measured by following the oxidation of NADH (Bottje et al., 2002). Mitochondria (200 μL, 100 μg of protein) were added to a solution containing 50 m*M* Tris-HCl and 0.1 m*M* 2,6-dichloroindophenol in a final volume of 3.25 mL. The reaction was initiated with addition of 50 μL of 14.1 m*M* NADH. Absorbance at 600 nm was monitored for 10 min to follow the rate of oxidation of NADH, and the enzyme activity

Table 1. Mitochondrial oxygen consumption for state 3 and state 4 respiration rates in brain mitochondria provided with glutamate-malate or succinate as an energy substrate isolated from Tibet chicken or Silky chicken embryos in a normoxic or simulated hypoxic hatchibator on d 16 of incubation[1]

Oxygen concentration	Chicken breed	Glutamate-malate		Succinate	
		State 3 (n = 10)	State 4 (n = 10)	State 3 (n = 10)	State 4 (n = 10)
		- (nmol O/min per mg of protein) -		- (nmol O/min per mg of protein) -	
Normoxia (21%)	Tibet	13.58 ± 1.16	3.80 ± 0.40[a]	10.03 ± 0.56[b]	5.71 ± 0.29
	Silky	12.95 ± 0.96	3.56 ± 0.32[a]	9.17 ± 0.65[b]	5.53 ± 0.48
Hypoxia (13%)	Tibet	15.97 ± 1.20	2.57 ± 0.19[b]	13.42 ± 1.46[a]	5.48 ± 0.61
	Silky	13.40 ± 1.21	2.68 ± 0.23[b]	13.43 ± 1.08[a]	5.49 ± 0.48

[a,b]Respiration values within a column with different letters are different ($P < 0.05$).

[1]Values represent the mean ± SE.

was determined by the change of absorbency. Values for complex I were expressed in units of changes of absorbency per minute per gram of mitochondrial protein.

Statistical Analysis

Means and standard errors were calculated for all variables in all groups, and means were separated by t-tests of Excel.xp (Microsoft Corp., Redmond, WA) and Duncan test of SPSS 13.0 (SPSS Inc., Chicago, IL). The significance levels were set at $P < 0.05$ and $P < 0.01$.

RESULTS AND DISCUSSION

Values for respiration rates for Tibet chicken and Silky chicken embryos are provided in Table 1. There were no differences between brain mitochondrial respiration of Tibet chicken and Silky chicken embryos for state 3 (active respiration in the presence of excess ADP) or state 4 (resting respiration when ADP becomes limiting) with glutamate-malate or succinate as an energy source under the normoxic or hypoxic condition. Respiration rates of embryonic mitochondria isolated from both chicken breeds were affected by oxygen concentration in hatchibators. With glutamate-malate as an energy source, state 4 respiration of embryos of both chicken breeds in the normoxic hatchibator was higher than that in the simulated hypoxic hatchibator ($P < 0.05$). States 3 respiration of embryos of the 2 breeds in the normoxic hatchibator was lower than that in the simulated hypoxic hatchibator when succinate was provided as an energy source ($P < 0.05$). In both chicken breeds, there were no differences between normoxic and hypoxic group in state 3 respiration with glutamate-malate as energy source, and in state 4 respiration when succinate was provided as energy source.

Under the normoxic incubation condition, there were no differences in brain mitochondrial respiratory function between Tibet chicken and Silky chicken embryos when brain mitochondria were provided with glutamate-malate or succinate as energy substrate (Figure 1a). Under the simulated hypoxic incubation condition, the RCR in Tibet chicken embryos was higher than that in Silky chicken embryos when brain mitochondria were provided with glutamate-malate ($P < 0.05$) (Figure 1b). The result indicated that electron transport was more tightly coupled in Tibet chicken than in Silky chicken embryonic brain mitochondria under the simulated hypoxic incubation condition. But there were no differences in the RCR in brain mitochondria provided with succinate between Tibet chicken and Silky chicken embryos incubated in the hypoxic environment (Figure 1b). These findings suggested that the difference in brain mitochondrial respiratory function between Tibet chicken and Silky chicken embryos under the hypoxic incubation condition might be due to differences in the function of complex I. There was also no difference in the ADP/O in brain mitochondria with glutamate-malate or succinate as energy substrate between Tibet chicken and Silky chicken embryos incubated in the same hatchibator (Figure 1a, 1b), which indicated that there was no obvious difference in the ability of the embryonic brain to carry out oxidative phosphorylation between the Tibet chicken and Silky chicken.

The RCR and the ADP/O in brain mitochondria of either breed embryos were higher in the simulated hypoxic hatchibator than those in the normoxic hatchibator with glutamate-malate or succinate as energy substrate (Figure 2a, 2b). These results indicated that electron transport was more tightly coupled and the oxidative phosphorylation was more effective in hypoxic than those in the normoxic group of embryos of either chicken breed.

There was no difference in complex I activity in brain mitochondria between normoxic and hypoxic embryos of Tibet chicken (Figure 3). In contrast, the complex I activity of hypoxic Silky chicken embryos was higher than that of normoxic embryos (Figure 3; $P < 0.01$). Under the normoxic incubation condition, there was no difference in complex I activity in brain mitochondria between Tibet chicken and Silky chicken embryos, whereas in the simulated hypoxic hatchibator, the complex I activity of brain mitochondria isolated from Silky chicken embryos was higher than that of Tibet chicken embryos (Figure 3; $P < 0.01$). These results showed that oxygen concentration had no effect on the complex I activity in brain mitochondria of Tibet chicken embryos, while hypoxia induced an increase in the complex I activity in brain mitochondria of Silky chicken embryos.

Changes of mitochondrial respiratory function in mouse (Magalhães et al., 2005) or rat (Costa et al., 1988; Song et al., 1999; Gao et al., 2000) after short-term or long-term hypoxia have been reported. It has also been

reported that the mitochondrial volume density of highland native was lower than that of lowland native (Kayser et al., 1991, 1996). However, the results obtained in the present study, to our knowledge, were the first to provide evidence of differences in embryonic mitochondrial respiratory function between the highland native animal and the lowland native animal of the same species.

In the present study, hypoxia affected both the mitochondrial phosphorylation efficiency and the coupling between respiration and ATP synthesis. The RCR and the ADP/O were higher in brain mitochondria of embryos of both breeds in the simulated hypoxic hatchibator with either energy substrate (Figure 2). Combining this result with state 4 and state 3 respiration rates (Table 1), we suggested that the increases in the RCR with glutamate-malate and succinate as energy substrates (Figure 2) were mainly due to a decrease in state 4 respiration and an increase in state 3 respiration in hypoxic embryos, respec-

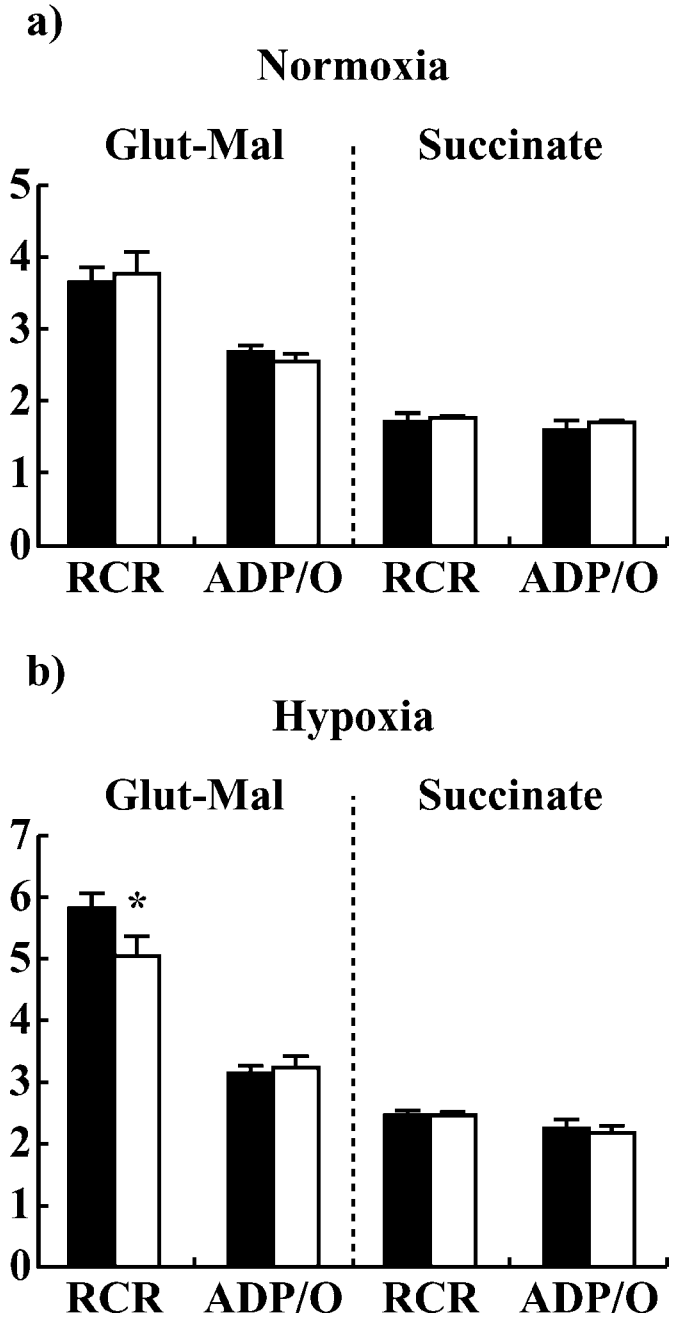

Figure 1. Mitochondrial respiratory function in brain mitochondria isolated from Tibet chicken (shaded bars) and Silky chicken (open bars) embryos incubated in a normoxic hatchibator (a) or a simulated hypoxic hatchibator (b). Functional measurements include the respiratory control ratio (RCR) and the adenosine 5′-diphosphate:oxygen ratio (ADP/O) for embryonic brain mitochondria provided with glutamate-malate (Glut-Mal) or succinate as energy sources. Each bar represents the mean ± SE for each group, and each group contains 10 samples. *The bar indicates that the parameter values are significantly different between Tibet chicken and Silky chicken embryos ($P < 0.05$).

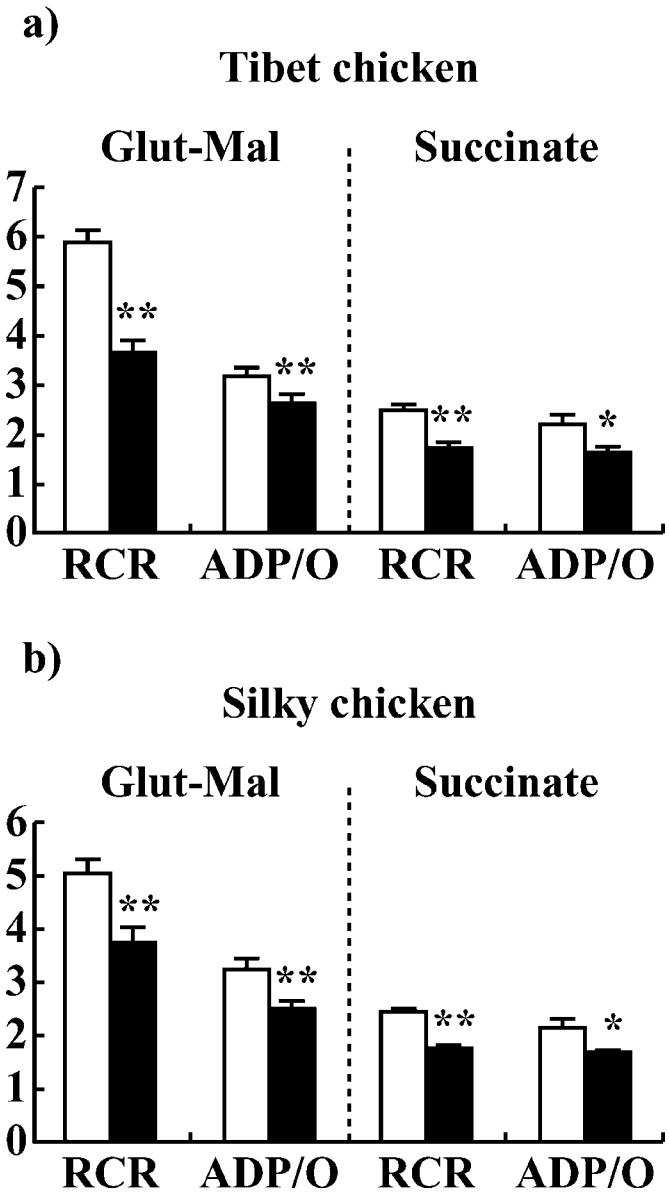

Figure 2. Mitochondrial respiratory function in brain mitochondria isolated from Tibet chicken (a) and Silky chicken (b) embryos incubated in a normoxic hatchibator (shaded bars) or a simulated hypoxic hatchibator (open bars). Functional measurements include the respiratory control ratio (RCR) and the adenosine 5′-diphosphate:oxygen ratio (ADP/O) for embryonic brain mitochondria provided with glutamate-malate (Glut-Mal) or succinate as energy sources. Each bar represents the mean ± SE for each group, and each group contains 10 samples. *Mean value for normoxic embryos is lower than hypoxic embryos ($P < 0.05$); **Mean value for normoxic embryos is lower than hypoxic embryos ($P < 0.01$).

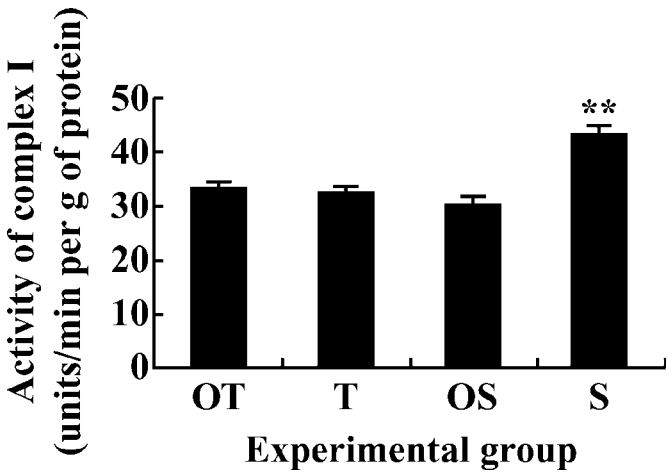

Figure 3. Activity (units/min per g of protein) of complex I in brain mitochondria obtained from Tibet chicken hypoxic (T) or normoxic (OT) embryos and Silky chicken hypoxic (S) or normoxic (OS) embryos. Each bar represents the mean ± SE for each group (n = 10 for each group). **The bar indicated that the parameter value of Silky chicken hypoxic group is significantly higher than other groups ($P < 0.01$).

tively. State 4 respiration and the ADP/O are associated in theory with the intrinsic and extrinsic uncoupling of oxidative phosphorylation (Hoch, 1992; Kadenbach, 2003), and we hypothesized that the decrease in state 4 respiration and the increase in ADP/O may imply a reduction of cytochrome c oxidase slip (Kadenbach, 2003), a change of proton permeability of inner mitochondrial membrane (Hoch, 1992), or both. With regard to state 3 respiration in brain mitochondria provided with glutamate-malate as energy substrate, our data also showed that hypoxic embryos possessed higher values compared with normoxic embryos of the same breed, despite the fact that differences were not significant (Table 1; $P = 0.38$ in Silky chicken; $P = 0.09$ in Tibet chicken). Because a decrease in state 3 respiration can be induced by the inhibition of complex I (Martínez et al., 2005) or ATPase (Wei et al., 1985) activity, we hypothesized that the increase in state 3 respiration in this study may imply an increased activity of ATPase or complex I, or both, of chicken embryos incubated in the hypoxic environment.

Under the normoxic incubation condition, there were no significant differences between Tibet chicken and Silky chicken embryonic brain mitochondria for all parameters in the present study. The result suggested that embryonic brain mitochondria isolated from Tibet and Silky chicken had the same mitochondrial phosphorylation efficiency and the same coupling ability when incubated in a normoxic environment, which agrees with the observation that Tibet and Silky chicken have the same hatchability in the normoxic hatchibator (our unpublished data). Under the hypoxic incubation condition, our result confirmed the difference in complex I activity between embryos of the 2 studied chicken breeds (Figure 3). The increase in complex I activity observed in hypoxia-exposed Silky chicken embryos may be a kind of physiological compensation and may be due to one or both of the following causes: 1) increased expression of complex I; 2) change of the state of reversible phosphorylation in complex I subunits (Scacco et al., 2000; Papa et al., 2002). Unlike Silky chicken embryos, Tibet chicken embryos had a relatively stable value of complex I activity whether they were incubated in the hypoxic or in the normoxic hatchibator (Figure 3). The Tibet chicken is a kind of highland native animal and has adapted itself to hypoxia. This result may reflect the difference in the relative hypoxic degree between Tibet chicken and Silky chicken embryos; that is, Silky chicken embryos were more sensitive to hypoxia than Tibet chicken embryos when they were incubated in the same simulated hypoxic hatchibator.

In summary, the results of this study provided evidence that electron transport in brain mitochondria of Tibet chicken embryos was more tightly coupled than that of lowland chicken (Silky chicken) embryos in a hypoxic incubation environment when glutamate-malate was provided as energy substrate. The higher RCR was associated with the difference in complex I activity between embryos of the 2 chicken breeds. Further studies are planned to investigate whether the differences in complex I activity in the embryonic brain between the Tibet chicken and the Silky chicken are caused by genetic variation of complex I subunit genes and to analyze the association of mitochondrial respiratory function with genetic polymorphism of complex I subunits.

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