《Research Note》

Ontogenic Profile of Gluconeogenic Key Enzyme Gene Expressions in Embryonic Chicken Liver and Muscle

Tushar K. Roy¹, Atsushi Iwasawa², Yasutake Shimizu², Koji Kageyama³ and Norio Yoshizaki²

¹ United Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, Japan

² Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan

³ River Basin Research Center, Gifu University, Gifu 501-1193, Japan

To elucidate biochemical mechanisms underlining high plasma glucose in chicken embryo, gene expression profile of gluconeogenic key enzymes were characterized. The liver and skeletal muscle were collected from 13- to 21-day embryos (E) ($n=8$) of layer chicken and the mRNA of pyruvate carboxylase (PC), cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C), mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M), muscle-type fructose-1,6-bisphosphatase (mFBPase), liver-type fructose-1,6-bisphosphatase (lFBPase) and glucose-6-phosphatase (G6Pase) were measured by real-time PCR. All the enzymes were expressed in the liver, whereas in the skeletal muscle, only mFBPase and G6Pase were detected. In the liver, all the enzymes except for G6Pase peaked either on E13 or E15. G6Pase expression was the highest between E15 and E19. In the skeletal muscle, mFBPase peaked on E19 and G6Pase peaked on E15. These results suggest that gluconeogenesis is active in chicken embryos but regulations of mRNA expressions of the gluconeogenic key enzymes are different between liver and muscle. The results also suggest that the skeletal muscle, which is generally not regarded as a tissue conducting an active gluconeogenesis, may contribute to the regulation of embryonic plasma glucose to some extent, and warrant further investigations of the presence of enzyme activities.

Key words: chicken, embryo, enzyme, gluconeogenesis, mRNA

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Introduction

Chickens maintain a plasma glucose level that is about twice as high as that of most non-ruminant mammals (Bell and Sturkie, 1965; Beuchat and Chong, 1998). Chickens fed with carbohydrate diet could easily maintain plasma glucose, but it is noteworthy that chicken embryos that depend only on the nutrient deposits in the egg yolk and albumen also have a high plasma glucose level. Previous reports (Yarnell *et al.*, 1966; Evans and Scholz, 1973; Lu *et al.*, 2007) demonstrate that the plasma glucose of chicken embryos already exceeds a non-ruminant mammal level in the second week of incubation and increases by the day of hatching to the level that is almost as high as that of adult chickens. The present authors are interested in how the embryos increase plasma glucose to the adult level using the finite nutrient deposits in the egg. The yolk consists of approximately 49% water, 17% protein, 33% lipid and 1% carbohydrate, whereas the albumen consists of approximately 88% water, 11% protein,

1% carbohydrate and trace of lipid (Romanoff and Romanoff, 1949). It is thus expected that gluconeogenesis is active in the embryo to maintain high plasma glucose (Pearce, 1971) and there is a body of literature where activities of gluconeogenic enzymes are measured during incubation of the chicken egg (reviewed in Pearce, 1971). However, to date, mRNA level study of gluconeogenic enzymes are few (Savon *et al.*, 1993; Yadgary and Uni, 2012). Enzyme activities *in vivo* is regulated complicatedly by such factors as allosteric regulators and interacting proteins (Stevens, 1996), and hence, not only enzyme activity experiments *in vitro* but also other experiments like assessment of enzyme gene expressions as well as transcriptional and post-transcriptional processes would help to better understand how the enzyme is actually working *in vivo*. Thus, the present study was undertaken to clarify mRNA expression of all the four enzymes (pyruvate carboxylase, EC 6.4.1.1, PC; phosphoenolpyruvate carboxykinase, EC 4.1.1.32, PEPCK; fructose-1,6-bisphosphatase, EC 3.1.3.11, FBPase; glucose-6-phosphatase, EC 3.1.3.9, G6Pase) that are known as the gluconeogenic key enzymes (Stevens, 1996) between day 13 and day 21 of chicken embryos. Since the reactions catalyzed by these four enzymes are virtually irreversible, they are the rate-

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Correspondence: A. Iwasawa, Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan. (E-mail: aiwa@gifu-u.ac.jp)

limiting enzymes of gluconeogenesis (Granner and Pilkis, 1990; Stevens, 1996; Jitrapakdee *et al.*, 2008). To our knowledge, this is the first report where mRNA expressions of all of the four key enzymes of gluconeogenesis in the chicken embryonic liver and muscle were analyzed. The overall results suggested a possibility of active gluconeogenesis in these tissues that could lead to high plasma glucose in chicken embryos.

Materials and Methods

Animals and Sampling

Fertile eggs of Lohman white layer chicken purchased from Ghen Corporation (Gifu, Japan) were incubated at 37.8 °C under 70% humidity. The start of the incubation was called day 0 (E0). Blood samples ($n=10$) were collected from E9 to E21 (before hatch) embryos and adult Lohman white layer chickens with heparinized syringes, centrifuged immediately and the plasma was stored at -80°C until measurement of glucose. The liver and skeletal muscle (thigh muscles) were collected from E13 to E21 (before hatch) embryos ($n=8$), snap frozen in liquid nitrogen and stored at -80°C until further processing. All experiments were performed in accordance with the Regulation for Animal Experiments in Gifu University.

Quantification of Plasma Glucose

Plasma glucose concentration was measured by glucose oxidase method using Glucose CII Test kit (Wako Pure Chemicals, Osaka, Japan) as recommended by the manufacturer.

RNA Isolation and Reverse Transcription

Total RNA was extracted from frozen liver and skeletal muscle using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Purity and concentration of the extracted RNA were assessed by using Nanovue spectrophotometer (GE Health-

care, Tokyo, Japan). Total RNA ($1\mu\text{g}$) was reverse transcribed into first strand cDNA by using ReverTra Ace (TOYOBO, Osaka, Japan) and random primer (TOYOBO) as recommended by the manufacturer.

Real-time PCR Analysis

The primers (Table 1) for real-time PCR were designed from previously reported *Gallus gallus* PC, PEPCK (both cytosolic and mitochondrial isozymes), FBPase (both liver and muscle isozymes), G6Pase and 18s ribosomal RNA (as a housekeeping gene) sequences in the GenBank database using Primer3 software (<http://frodo.wi.mit.edu/>). Real-time PCR amplification was performed by the ABI prism 7000 apparatus (Applied Biosystems, Tokyo, Japan) using SYBR Premix Ex Taq II (Perfect Real Time) (Takara Bio, Shiga, Japan). A reverse-transcribed product ($2\mu\text{l}$) was added to a $20\mu\text{l}$ mix containing $10\mu\text{l}$ SYBR Premix EX Taq II (2x), $0.4\mu\text{l}$ of ROX reference dye (50x) and $6\mu\text{l}$ water. The cycling condition was 40 cycles at 95°C for 5 sec, 60°C for 20 sec and 72°C for 31 sec after initial 30 sec incubation at 95°C .

The amplification program was followed by a dissociation curve analysis to detect any non-specific amplification. Each experimental and standard sample was assayed in duplicate. Relative expression values were calculated based on the standard curve method using a serial dilution of pooled cDNA as standard. The amplified products of PCR were electrophoresed and sequenced to confirm the specificity of the amplification.

Statistics

Data were presented as means $\pm$ SEM. Comparisons among groups were performed with one way ANOVA followed by Tukey's multiple comparison test using SPSS statistical package (ver. 13.0). Differences were considered statistically significant at $p < 0.05$.

Table 1. Primers used for real-time PCR of gluconeogenic key enzymes

Enzyme	Primer sequence (5'-3')	Amplicon length (bp)	GenBank accession No.
PC	TGGGGCATAAATTCAAGGAG GAATTCGACACGGAGAGAG	194	AF509529.1
PEPCK-C	TGCTGGTGTGCCTCTTGAT CACACGGGAATTCTCTCCAT	295	M14229.1
PEPCK-M	GTTACCTGGAACATTGGCTGTC CAGGTCCAAATCCCCTTCTT	224	J05419.1
mFBPase	AACTACAGGGACAGAGGCG AAGTGGGTGTTGAAATGCTA	228	XM_425039.2
lFBPase	CGGGAGATCAGGTGAAGAAA ATGGTCCCAATGGAACAAG	208	XM_425040.2
G6Pase	CATGTACTTCACTTCTTCTCC TATTGTATCAGCGTGGCGTA	137	XM_422017.2
18s rRNA (as reference)	TCAACTTTTCGATGGTACTGTCTGTG CTTGGATGTGGTAGCCGTTTCT	106	AF173612.1

Enzymes: PC, pyruvate carboxylase; PEPCK-C, cytosolic isozyme of phosphoenolpyruvate carboxykinase; PEPCK-M, mitochondrial isozyme of phosphoenolpyruvate carboxykinase; mFBPase, muscle isozyme of fructose-1,6-bisphosphatase; lFBPase, liver isozyme of fructose-1,6-bisphosphatase; G6Pase, glucose-6-phosphatase.

Fig. 1. **Developmental changes in plasma glucose of chicken embryo.** Values are means $\pm$ SEM of 10 samples. Different letters indicate significant differences by Turkey's multiple range test ($p < 0.05$).

Results

Figure 1 shows changes in plasma glucose concentration during incubation. On E9, plasma glucose was already 132 mg/dl, increased significantly on E15 (162 mg/dl) and E18 (200 mg/dl) when it reached adult level. Figure 2 shows relative gene expressions of the gluconeogenic key enzymes in the liver and skeletal muscle between E13 and E21. All the enzymes were expressed in the liver, whereas in the skeletal muscle, only the muscle isozyme of FBPase (mFBPase) and G6Pase expressions were above the detectable level. In the liver, relative expressions of all the enzymes except for G6Pase peaked either on E13 or E15 and decreased thereafter. G6Pase expression was the highest between E15 and E19. In the skeletal muscle, mFBPase peaked on E19 and G6Pase peaked on E15.

Discussion

Liver

There have been many studies where the activities of the gluconeogenic key enzymes in embryonic chicken liver are investigated (Rinaudo, 1961; Ballard and Oliver, 1963; Nelson *et al.*, 1966; Felicioli *et al.*, 1967; Evans and Scholz, 1973; Gonzalez-Mujica *et al.*, 2001). They vary considerably in experimental design, but the enzymes showed maximum activities largely between E14 and E17 in some of these reports (Rinaudo, 1961; Ballard and Oliver, 1963; Nelson *et al.*, 1966; Felicioli *et al.*, 1967). These results are consistent in tendency with our present data that the expressions of the enzymes had peaks mainly on E13 or E15 and declined thereafter (Fig. 2).

In birds as well as in mammals, two PEPCK isozyme forms are known, one located in the cytosol (PEPCK-C), the other in the mitochondria (PEPCK-M) (Hod *et al.*, 1986). In most mammals except for rodents, both the liver and kidney contain the both type of isozymes, whereas in birds, it is well

known that the liver basically contains PEPCK-M only and the kidney contains the both isozymes (Shen and Mistry, 1979a; Shen and Mistry, 1979b; Watford *et al.*, 1981). Due to the reason of intracellular translocation of reducing equivalents or NADH between the cytosol and the mitochondria, tissues possessing no PEPCK-C (like chicken liver) are limited in their capacity of gluconeogenesis from pyruvate, while gluconeogenesis from lactate is not affected by the location of PEPCK (Watford *et al.*, 1981; Hod *et al.*, 1986). García *et al.* (1986) reported that lactate in embryonic chicken muscle increased drastically from E15 until hatch. Høiby *et al.* (1987) estimated that the net content of lactate in the allantoic fluid, the amniotic fluid and the yolk increased between E14 and E20 by 48% in total. Lactate increased in these tissues could be released into circulation, transported to the liver (Cori cycle), and the high expression of hepatic PEPCK-M that peaked on E13 and E15 (Fig. 2) may contribute to metabolize this increased lactate in coordination with PC and FBPase that are also expressing high (Fig. 2).

Our results clearly showed that PEPCK-C expression was also present in the liver on E13 and E15 but very low on and after E17 (Fig. 2), which is in accordance with a Northern blot analysis by Savon *et al.* (1993) who reported that PEPCK-C gene expression was present in embryonic chicken liver but gradually decreased toward hatch. Also, PEPCK-C gene expression in embryonic chicken liver was inducible by dexamethasone administration with a progressively decreasing responsiveness from E12 until E15 (McCaffrey and Hamilton, 1994). However, Felicioli *et al.* (1967) reported that enzyme activity of PEPCK-C in the liver was very low between E13 and E21. Watford (1985) pointed out that, although PEPCK-C mRNA was considerably increased in the liver of adult chickens deprived of food, its enzyme activity remained very low. It is therefore possible that the translation of PEPCK-C mRNA is virtually negligible in the liver not only of adult but also of embryonic chickens.

Fig. 2. Developmental changes in mRNA expression of gluconeogenic key enzymes in the liver and skeletal muscle from chicken embryo. Key enzymes: PC, pyruvate carboxylase; PEPCK-C, cytosolic isozyme of phosphoenolpyruvate carboxykinase; PEPCK-M, mitochondrial isozyme of phosphoenolpyruvate carboxykinase; mFBPase, muscle isozyme of fructose-1,6-bisphosphatase; lFBPase, liver isozyme of fructose-1,6-bisphosphatase; G6Pase, glucose-6-phosphatase. The relative mRNA quantities of these enzymes are expressed as means $\pm$ SEM of 8 embryos. Different letters indicate significant differences within the same enzyme by Turkey's multiple range test ($p < 0.05$).

In mammals, liver isozyme of FBPase (lFBPase) is expressed predominantly in the liver and kidney, and muscle isozyme (mFBPase) in the muscle and intestine (Tillmann *et al.*, 2000; Tillmann *et al.*, 2002), and hence, they are localized in several tissues regardless of their name. The tissue specificity of chicken l/mFBPase that was revealed in part in

the present study (Fig. 2) and their possible different roles have not reported so far and deserve further studies.

The PC was first isolated from avian liver homogenate and many studies have revealed its complicated mechanisms of regulation in transcription, translation and post-translation (Jitrapakdee *et al.*, 2002). In the liver of E18 chicken em-

bryo, mRNA expression was detected by Northern analysis (Jitrapakdee *et al.*, 2002), but its change in the course of incubation has not been determined until the present study.

While the present study was going on, Yadgary and Uni (2012) investigated mRNA expressions of PEPCK-M, PEPCK-C, IFBPase and G6Pase in the chicken ($n=4$) liver between E13 and E21. Although their experimental design are generally the same as ours, the embryonic ages showing highest expressions are different: E19–E21 (Yadgary and Uni, 2012) vs. E15 (present study) in PEPCK-M, E13 vs. E15 in PEPCK-C, E17 vs. E13 in IFBPase and E20 vs. E15–E19 in G6Pase gene expressions. The reason for these discrepancies could be the difference between broiler (Yadgary and Uni, 2012) and layer (present study) chickens. Modern broilers and layers have been thoroughly selected for different purposes and it is reported that, already in embryonic ages, broiler and layer chickens are considerably different in some developmental parameters such as yolk weight, growth rate, heat production, lipid metabolism, protein turnover and various hormone concentrations (Muramatsu *et al.*, 1990; Sato *et al.*, 2006; Everaert *et al.*, 2008).

Skeletal Muscle

In the skeletal muscle, only mFBPase and G6Pase gene expressions were detected (Fig. 2), which is in accordance with the present knowledge that amongst four gluconeogenic key enzymes only FBPase (purified protein from adult chicken: Annamalai *et al.*, 1977) and G6Pase (enzyme activity in post-hatch chick: Asotra, 1986) are present in the skeletal muscle of chickens. Metabolic implications of FBPase and G6Pase in the muscle have long been blurred because the muscle, lacking in PC and PEPCK, is theoretically unable to carry out gluconeogenesis from such materials as pyruvate and lactate (Stevens, 1996). However, since the reversibility of a *glycolytic* key enzyme pyruvate kinase has been suggested in mammals (Dyson *et al.*, 1975; Dobson *et al.*, 2002), it is postulated that mammalian muscle is able to carry out gluconeogenesis from lactate even without PC and PEPCK (Dzugaj, 2006). Lactate in embryonic chicken muscle is reported to increase drastically from E15 until hatch (García *et al.*, 1986). The expression of mFBPase that increases from E15 until E19 (Fig. 2) may thus contribute to the gluconeogenesis from the increased lactate within the muscle.

In mammals, a catalytic subunit gene of G6Pase was proved to be ubiquitously expressed including in the muscle (Martin *et al.*, 2002; Guionie *et al.*, 2003; Shieh *et al.*, 2003; Shieh *et al.*, 2004), and G6Pase protein and enzyme activity were proved in the muscle (Hirose *et al.*, 1986; Watanabe *et al.*, 1986; Shieh *et al.*, 2004). This has raised a possibility of contribution of the skeletal muscle to mammalian glucose homeostasis by releasing glucose into circulation (Shieh *et al.*, 2003; Shieh *et al.*, 2004). We speculate from the data of G6Pase mRNA (Fig. 2) that the embryonic chicken skeletal muscle also releases glucose into circulation.

Organ-specific Contribution to the Plasma Glucose Level

Plasma glucose was already above 100 mg/dl (a typical level of non-ruminant mammals) on E9, gradually increased

thereafter and reached adult level on E18 (Fig. 1). Although previous reports on embryonic blood glucose (Yarnell *et al.*, 1966; Evans and Scholz, 1973; Lu *et al.*, 2007) and the present result are all different from each other in glucose concentrations and detailed pattern of their changes during incubation possibly due to the method of glucose measurement or the strain and the nutritional status of the mother hen and the embryo, these reports are consistent in that embryonic plasma glucose level is in increasing tendency toward hatch. Our data (Fig. 2) showed that G6Pase was expressed both in the liver and muscle throughout the experimental period, which suggests possible contribution of the both organs to the increase of plasma glucose by releasing glucose into circulation, although the expression was not simply parallel to the increasing pattern of plasma glucose.

In the liver, PC, PEPCK, and FBPase showed maximum expressions either on E13 or E15 (Fig. 2), when liver glycogen content is in the course of rapid increase (Ballard and Oliver, 1963; Romanoff, 1967; Yadgary and Uni, 2012). On the other hand, liver G6Pase expression was the highest between E15 and E19. Thus, in terms of gene expression for gluconeogenesis, it may be postulated that glycogen synthesis from lactate (as described in the liver section) is most activated around E13–E15, whereas the release of glucose, which could be metabolized from lactate or converted from the stored glycogen, continues until before hatch.

In the skeletal muscle, G6Pase expression was the highest on E15 (Fig. 2). It is thus possible that the muscle contributes to the increase of blood glucose especially around this age. Glycogen content of the skeletal muscle is reported to increase from E10, reaching the highest on E19–E20 (Romanoff, 1967; García *et al.*, 1986). Muscle FBPase whose expression reached the highest on E19 (Fig. 2) would thus contribute to accumulate glycogen from lactate (as described in the muscle section) in the muscle in preparation for the energy demand on hatching.

Between the establishment of the chorioallantois (about E8) and 2–3 days before internal pipping (peep inside the eggshell before hatch), the embryos are under sufficient oxygen supply, and hence, the energy necessary for development, growth and differentiation of the embryo can be generated by β -oxidation of free fatty acids mobilized from yolk lipid (De Oliveira *et al.*, 2008). The present results suggest a possibility that, meanwhile in the liver and skeletal muscle, gluconeogenesis is active and contribute to the gradual elevation of blood glucose to the adult level and also to accumulate glycogen reserves for the energy demand for the last phase of incubation leading to hatch.

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