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Mechanisms Underlying High Blood Glucose Levels in Laying Hens

メタデータ	言語: English
	出版者:
	公開日: 2014-04-20
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	キーワード (En):
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URL	http://hdl.handle.net/20.500.12099/46775

Mechanisms Underlying High Blood Glucose Levels in Laying Hens

（産卵ニワトリの糖代謝とその制御機構に関する研究）

２０１３年

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Abbreviations

E9 = Embryonic day 9 (E means embryonic day)

HK – Hexokinase

GK – Glucokinase

GLUTs – Glucose transporters

PC – Pyruvate Carboxylase

PEPCK – Phosphoenolpyruvate Carboxykinase

lFBPase – Liver isoenzyme of Fructose-1,6-bisphosphatase

mFBPase –Muscle isoenzyme of Fructose-1,6-bisphosphatase

G6Pase – Glucose-6-phosphatase

IRC – Immunoreactive cells

RACE = Rapid amplification of cDNA ends

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General Introduction

Chickens maintain a blood glucose level that is twice as high as that in most mammals (Hazelwood and Lorenz, 1959; Belo *et al*., 1976). Chickens fed with high carbohydrate diet could easily maintain plasma glucose, but it is noteworthy that the incubating chicken embryos which depend only on the nutrient deposits in the egg yolk and albumen also have a high plasma glucose level. The yolk consists of approximately 49% water, 17% protein, 33% lipid and 1% carbohydrate, whereas the albumen consists of 88% water, 11% protein, 1% carbohydrate and trace of lipid (Romanoff and Romanoff, 1949). That small amount of carbohydrate is used by embryonic day 7 (E7) in the chicken (Bate and Dickson, 1986), and thus the main source of energy throughout the incubation is lipid and protein. In spite of this, plasma glucose is reached a high level by the second week of incubation and increased by the day of hatching to the level that is almost as high as that of adult (Yarnell *et al*., 1966; Evans and Scholz, 1973; Lu *et al*., 2007). This dissertation is aimed to clarify the mechanisms underlying high blood glucose in laying hens by investigating the mechanisms underlying high blood glucose level during the last half (E11 to E21) of embryonic development because the developing chicken embryos seems to be an excellent model as they developed in a closed system (eggs) and therefore effects of food intake need to be taken into consideration.

During the last half of the embryonic development of chicken, glucose homeostasis mechanism is complex. During that period, embryo needs to produce more glucose for maintaining high blood glucose level and rapid growth (Moran, 2007). On the other hand, embryo needs to store glycogen in liver and muscle for the preparation of hatching (Hazelwood, 1971). It is thus expected that gluconeogenesis is active during that period (Pearce, 1971).

Liver, muscle and kidney are important organs for glucose homeostasis. In liver, the

gluconeogenic capacity is well developed by E13, but in kidney it is only fully developed after hatching (Stevens, 2004). Along with developing enzymatic regulation, endocrine system starts developing very early during incubation. Insulin and glucagon is detected on E3 in chicken embryo. Glucagon is believed to play key roles for regulating plasma glucose in adult chicken instead of insulin as chicken lacks insulin responsive glucose transporter (Stevens, 2004). There must be a close association among hormone levels, enzymatic regulation of different metabolic pathway and metabolites in plasma.

Overall aim of research

The overall aim of this research was to investigate the effect of insulin and glucagon on enzymatic regulation (at mRNA level) of plasma glucose by investigating ontogeny of hexokinase isoenzymes and gluconeogenic key enzymes in liver, muscle and kidney and by observing ontogeny of insulin and glucagon immunoreactive cells in pancreas from E11~E21.

Research hypothesis

I hypothesized that the enzymatic regulation of glucose homeostasis in chicken embryo is a little different with adult chicken in some stages of development and muscle may release some glucose into blood. Glucagon has more effect on glucose homeostasis (especially on gluconeogenesis) than insulin, not only in the adult chicken but also in the chicken embryo.

Specific experimental objectives

1. To observe the ontogenic profile of hexokinase and glucokinase mRNA expressions in embryonic chicken liver and muscle using semi-quantitative PCR. The study revealed that muscle may play liver-like role in early incubation period by expressing glucokinase (although further study is required).
2. To observe the ontogenic profile of gluconeogenic key enzyme gene expressions in

embryonic chicken liver, muscle, and kidney by real-time PCR. By this study it is understood that before and after E15, gluconeogenesis is increased in liver and kidney. Moreover, muscle may release some glucose along with liver and kidney.

3. To observe the ontogenic profile of insulin and glucagon immunoreactive cells in embryonic chicken pancreas by using immunohistochemistry. By this study, it is understood that glucagon is in high concentration before and after E15 and may induce gluconeogenesis in liver, muscle and kidney. Insulin has secondary effect on glucose homeostasis.

Chapter 1 Literature review

Plasma glucose in birds and mammals

Glucose is a simple carbohydrate and is the most important sugar in human metabolism. The central role of glucose in metabolism arose early in evolution, and this glucose remains the nearly universal fuel and building block in modern organisms, from microbes to humans. And plasma glucose is a term used to refer to the amount of glucose in the plasma. This plasma glucose is the primary source of energy for the body's cells which is tightly regulated. Failure to maintain plasma glucose in the normal range leads to conditions of persistently high (hyperglycemia) or low (hypoglycemia) blood sugar. Diabetes mellitus, characterized by persistent hyperglycemia of several causes, is the most prominent disease related to failure of blood sugar regulation. Plasma glucose concentrations have been measured in quite a wide range of birds and it is observed that they all maintain high plasma glucose compared with mammals (Table 1).

Table 1. **Blood glucose concentrations in different avian species and mammals**

Species	**Order**	**Plasma glucose concentration (mg/dl)**
Horned lark	Passeriformes	445
Adelie penguin	Sphenisciformes	286
Bald eagle	Falconiformes	266
Spoonbill	Ciconiiformes	265
Domestic fowl	Galliformes	200
Quail	Galliformes	193
Mammals		45 ~ 149

Ref: Stevens, 2004

Birds could maintain those plasma glucose concentrations during fasting also as they are resistance to fasting hypoglycemia (Hazelwood, 1972). For that resistance, regular fasting in birds in connection with breeding, migration, or drastic climatic conditions does not obstruct bird normal life leading. In domestic fowl and quail plasma glucose concentrations are maintained during 3 days fasting (Davidson and Langslow, 1975; Didier et al., 1981). In a small passerine bird such as the horned lark, a 20 h fast reduces the liver glycogen content by ~ 90%, but the blood glucose concentration changes insignificantly from 24.7 ± 1.84 mM to 22.9 ± 1.56 mM (mean ± SEM) (Swain, 1992). Reduction of glucose utilization, together with high recycling of glucose carbon atoms might contribute to the sparing of glucose (Annison *et al*., 1966; Belo *et al*., 1976). Plasma glucose maintenance might also be related to enhance gluconeogenesis (Veiga *et al*., 1978), though an opposite response, probably related to diet, was observed in the black vulture, a carnivorous bird (Veiga *et al*., 1978). The blood glucose level not only depends on its turnover rate which depends on its rate of synthesis and utilization but also depends on its supply and demand. The utilization and demand vary between organs.

Role of different organs

In mammals, the liver is the major metabolic regulatory organ. About 90% of all circulating glucose not derived directly from the diet comes from the liver. The liver contains significant amounts of stored glycogen available for rapid release into circulation, and is capable of synthesizing large quantities of glucose from substrate such as lactate, amino acids, and glycerol, released by other tissues. In addition to controlling plasma glucose, the liver responsible for synthesis and release of the lipoproteins that adipose and other tissues use as the source of cholesterol and free fatty acids. During prolonged starvation, the liver is the source of both glucose and the ketone bodies required by the brain to replace glucose and the ketone bodies required by the brain to replace glucose. The liver uses glycolysis primarily as a source of biosynthetic intermediates, with amino acid and fatty acid breakdown providing the

majority of its fuel. Like the liver, the kidney has the ability to release glucose into the blood. Under normal conditions gluconeogenesis in the kidney provides only a small contribution to the total circulating glucose; however, during prolonged starvation, the kidney contribution may approach that of the liver. Kidney function is critical for glucose homeostasis for another reason; plasma glucose continuously passes through the kidney and must be efficiently reabsorbed to prevent disease. In mammals, the muscle release glucose into circulation or not is still blurred because but its ability to rapidly increase its glucose uptake is critical for dealing with sudden increases in plasma glucose. Recently, a catalytic subunit gene of G6Pase was proved to be expressed in mammalian muscle (Martin *et al*., 2002; Guionie *et al*., 2003). In exception with mammals, chicken muscle have glucose-6-phosphatase and fructose-1,6-bisphosphatase. It is perhaps surprising that the breast muscle of domestic fowl is a good source of fructose-1,6-bisphosphatase. Skeletal muscle has an additional role in maintaining plasma glucose levels: it releases free amino acids into circulation to serve as substrates for liver gluconeogenesis. The muscle can use glucose, fatty acids, and ketone bodies for energy. The muscle normally maintains significant amount of stored glycogen, small amounts of fatty acids, and contains a large pool of protein that can be broken down in emergencies. The resting muscle uses fatty acids as its primary energy source; however, glucose (from its own glycogen stores and from circulation), is preferred for rapid energy generation. The adipose tissue is the major site of fatty acid storage. Fatty acids are stored in the form of triacylglycerol, which is synthesized in the adipose tissue from glycerol-phosphate and free fatty acids. The glycerol-phosphate used must be derived from glycolysis in the adipose tissue; free glycerol cannot be phosphorylated because adipocytes lack the relevant kinase. In conclusions when liver gluconeogenesis is necessary the adipose tissue supplies free fatty acids and glycerol to the circulation to be taken up by the liver as substrate.

Glycolysis and hexokinase isoenzymes

Glycolysis (from *glycose*, an older term for glucose + *-lysis* degradation) is the metabolic pathway that converts glucose $C_6H_{12}O_6$, into pyruvate, CH_3COCOO^- + H^+ appears to be a universal system for liberation of energy, some of which is conserved in the high-energy compounds ATP (adenosine triphosphate) and some of which is NADH (reduced nicotinamide adenine dinucleotide). It occurs, with variations, in nearly all organisms, both aerobic and anaerobic. The wide occurrence of glycolysis indicates that it is one of the most ancient known metabolic pathways. It occurs in the cytosol of the cell. The pathway is sometimes known as the *Embden-Meyerhof-Parnas (EMP pathway)*, although the reactions involved in the conversion of glucose to pyruvate are very similar in all organisms, the fate of the pyruvate depends upon the organism and its environment. Glycolysis is a definite sequence of ten reactions involving ten intermediate compounds (one of the steps involves two intermediates). The intermediates provide entry points to glycolysis. For example, most monosaccharides, such as fructose, glucose, and galactose, can be converted to one of these intermediates. The intermediates may also be directly useful. For example, the intermediate dihydroxyacetone phosphate (DHAP) is a source of the glycerol that combines with fatty acids to form fat.

The reactions of glycolysis occur in two stages:

Stage I: A preparatory stage is which the hexose glucose is phosphorylated and cleaved to yield two molecules of the triose glyceraldehyde-3-phosphate. The process utilizes two ATPs in a kind of energy investment.

Stage II: The two molecules of glyceraldehyde-3-phosphate are converted to pyruvate, with concomitant generation of four ATPs.

The overall reaction is

Glucose + NAD^+ + 2ADP + 2Pi ⟶ 2NADH + 2 Pyruvate + 2ATP + $2H_2O$ + $4H^+$

The control of glycolysis is necessary to ensure that carbohydrate is degraded only as rapidly as energy is required by the cell. The requirement for energy will vary from organism to organism and form tissue to tissue. In the case of microorganisms, the synthetic processes involved in growth and replication will largely determine the need for ATP synthesis and the rates of these processes must be adapted to the nature of the environment or medium in which the microorganism is growing. In the glycolytic pathway, there are two rate limiting steps; one is catalyzed by hexokinase isoenzymes another is by pyruvate kinase isoenzymes.

Hexokinase

Glucokinase and hexokinase regulate the metabolism of glucose once it has entered a tissue and therefore, exert an effect on its uptake, thereby playing a key role in tissue intermediary metabolism.

Glucose + ATP → Glucose-6-phosphate + ADP + H^+

The large negative free energy change is indicative of an enzyme not operating near equilibrium, and, therefore, forming a rate-limiting step and an important potential control point. Hexokinase has been studied in a wide variety of mammalian tissues and has been found to exist as four isoenzymes (HKI, HKII, HKIII and HKIV), where they form a family of closely related enzymes encoded by unique genes. The other name of hexokinase IV is glucokinase (GK). Among them, HK-I mRNA is widely distributed in all vertebrate tissues and has high affinity for glucose. On the other hand HK-II is predominantly expressed in insulin sensitive tissues like muscle and adipose tissues. HK-1 seems to be coupled with GLUT1 and HK2 with GLUT4 (Ebeling *et al.,* 1998). Hexokinase III is predominantly expressed in spleen and lymphocyte and substrate-inhibited by glucose (Preller *et al.,* 1992). HK-IV (Glucokinase) has the most distinctive properties with half molecular mass (50 kDa) of other HKs (100 kDa) and low affinity for hexoses (Km 0.5~10 mM). HKI and HKII in most cells follow the Michaelis-Menten kinetics and in contrast GK displays sigmoidal

kinetics. Glucokinase has the most distinctive properties. It has narrower substrate specificity, effectively restricted to glucose, it does not show product inhibition by glucose-6-phosphate and it becomes saturated at much higher glucose concentrations. Glucokinase has only be found in the hepatocytes and the β-cells of the pancreas, both of which are served by the hepatic portal vein, which carries glucose absorbed by the small intestine. When glucose concentration in the hepatic portal vein is high, liver glucokinase regulates the uptake of glucose into the liver where it is converted into glycogen for storage. With the high blood glucose concentrations found in birds, one might expect a homologous glucokinase to be present in avian hepatocytes. A number of studies were made between 1963 and 1978 to detect glucokinase in the liver of domestic fowl, but reports appeared contradictory, some detecting its presence and others failing to do so. One of the difficulties lay in factors that interfere with the coupled spectrophotometric assay, which was generally used to detect it. This was overcome by using a radiochemical assay that measures directly the U-^{14}C glucose-6-phosphate formed, thus anabling glucokinase to be detected in the livers of the domestic fowl, the Mallard duck and pigeon (Stanley *et al*., 1984).

Apart from the studies specifically on GK mentioned above, hexokinase activity has been measured in cultured chick embryo hepatocytes (Hamer and Dickson, 1990) and in liver homogenate from goose, duck quail, lapwing, kelp gull, dove, parakeet, mocking bird, cow bird, black bird and yellow finch (Ureta *et al*., 1973) and in the pectoral muscles of American goldfinch, pigeon and domestic fowl (Yacoe and Dawson, 1983; Blomstrand *et al*., 1983).

Pyruvate kinase

The second rate limiting step in glycolysis is the conversion of phosphoenol pyruvate to pyruvate, catalyzed by pyruvate kinase. There are a number of isoenzymes of pyruvate kinase and they have been classified according to their properties and their tissue distribution in mammals. All four isozymes are tetramers.

Gluconeogenesis and its key enzymes

Gluconeogenesis is the pathway of formation of glucose from the nocarbohydrate precursor.

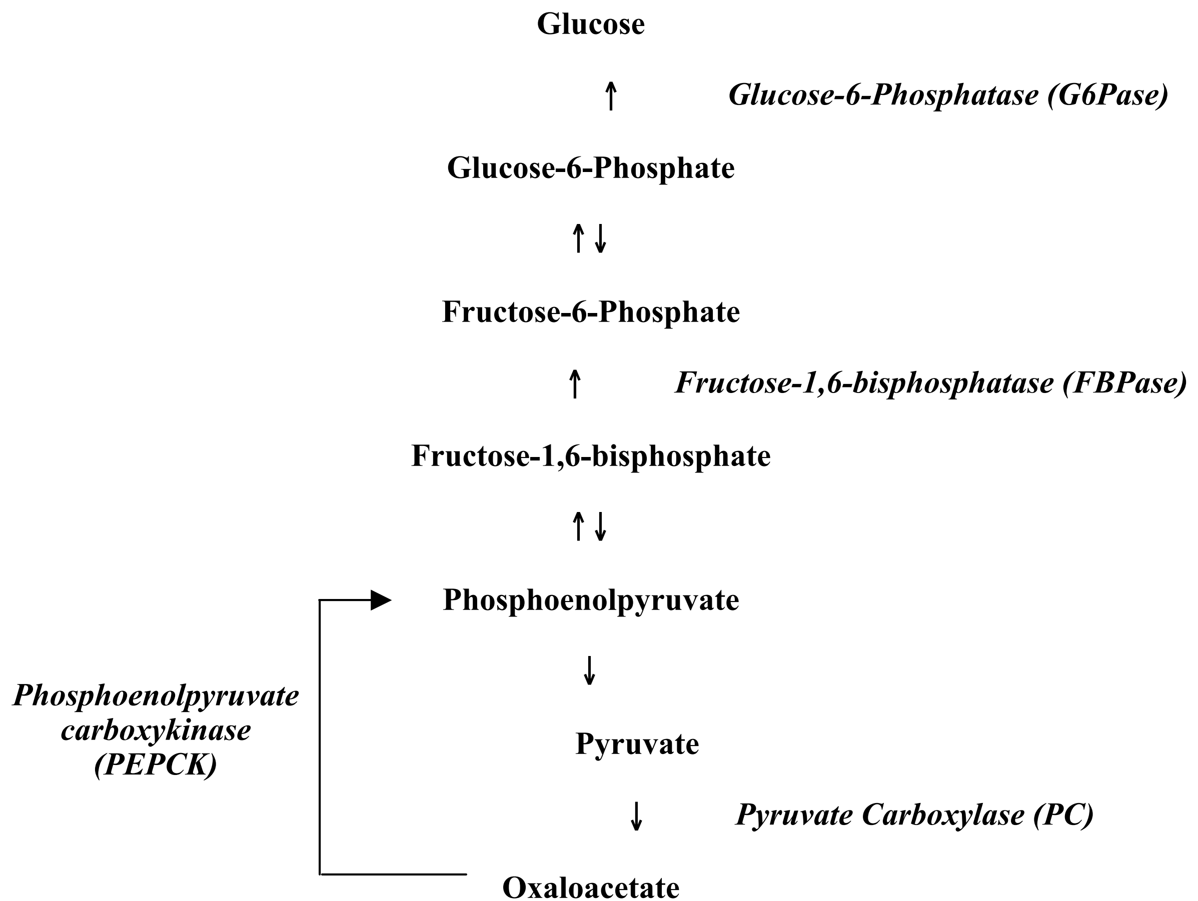


Fig 1. **Gluconeogenesis and its key enzymes**

The important precursor for gluconeogenesis in animals is three carbon compounds such as lactate, pyruvate, and glycerol as well as certain amino acids like aspartate, alanine, glutamate, serine and cysteine. The gluconeogenic amino acids are generally transaminated, or deaminated either directly or indirectly into pyruvate, oxaloacetate or oxoglutarate. Gluconeogenesis occurs in all animals, plants, fungi, and microorganism. Till to date it is told that gluconeogenesis takes place mainly in the liver and to a laser extent in renal cortex in animal. The reactions are essentially the same in all species.

Several enzymes are common to both glycolytic and gluconeogenic pathways, but 4 enzymes catalyse steps that only occur in gluconeogenesis: pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase and glucose-6-phosphatase. They catalyse the rate limiting steps in the pathway and are important control points. The metabolic steps in gluconeogenesis occur in two intracellular compartments: the cytosol and the mitochondrial matrix.

Pyruvate Carboxylase

Pyruvate carboxylase is a biotin-requiring enzyme which present in the mitochondrial matrix. Lactate present in the cytosol, is oxidized to pyruvate and enters the mitochodrail matrix where it converts into oxaloacetate by pyruvate carboxylase. Pyruvate carboxylase is allosterically activated by acetyl coenzyme A. This enzyme is mainly available in the liver and kidney. This enzyme is also available in other tissue but in lower activity. Its kinetic mechanism has been studied in detail using the enzyme purified from domestic fowl liver among the avian species.

Phosphoenolpyruvate carboxykinase (PEPCK):

PEPCK catalyzes one of the rate-controlling step of gluconeogenesis. It converts the oxaloacetate into phosphoenolpyruvate.

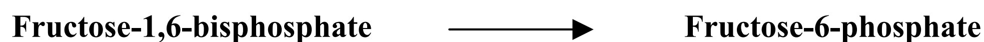
The enzyme has therefore been thought to be essential in glucose homeostasis, as evidenced by laboratory mice that contracted diabetes mellitus type 2 as a result of the over expression of PEPCK.

Avian kidney has two distinct PEPCK isoenzymes, mitochondrial and cytosolic PEPCK and so differs from avian liver which only has the mitochondrial PEPCK. The two avian isoenzymes of PEPCK have been purified and their cDNAs sequenced. There is 60% homology in their cDNAs. It is clear that these are the products of distinct genes, although there are similarities particularly in their binding sites. Another important difference is that

whereas the mitochondrial enzyme is constitutive, the cytosol PEPCK is able to adapt in response to dietary or hormonal stimuli (Watford, 1985). It has been believed that skeletal muscle is unable to carry out gluconeogenesis, since it lacks PEPCK. PEPCK, has a different intracellular distribution in avian liver compared with mammalian liver. PEPCK in both pigeon and domestic fowl liver is present almost exclusively (99%) in mitochondria (Soling *et al*., 1973) whereas in most mammals that have been studied, it is present mainly in the cytosol.

Fructose-1,6-bisphosphatase

Fructose-1,6-bisphosphatase is an enzyme in the liver, kidney and skeletal muscle that converts fructose-1,6-bisphosphate to fructose-6-phosphate in gluconeogenesis.



It is present in the cytosol and does the opposite job as phosphofructokinase, and both of these enzymes only work in one direction. Fructose-1,6-bisphosphatase has been purified from chicken breast muscle (MacGregor *et al*., 1982) and turkey liver (Han and Johnson, 1982). The enzyme shows hyperbolic kinetics with the substrate but is inhibited by AMP and high concentrations of substrate fructose-1,6-bisphosphate.

Glucose-6-phosphatase

Glucose-6-phosphatase catalyses the following reaction:

It enables the hexose moiety from phosphorylated glucose to be released into the bloodstream. It is present in the endoplasmic reticulum of liver and kidney, but activity in brain and skeletal muscle is generally low. Higher glucose-6-phosphatase activities have been detected in the liver from domestic fowl, duck, turkey, pigeon, hummingbird and black vulture and also in domestic fowl kidney (Nordlie, 1974). Low glucose-6-phosphatase activities have been detected in the pectoral muscles of domestic fowl, pigeon and house sparrow (Lackner *et al*., 1984). This may be an indication that small amounts of glucose can

be released from muscle for use in other tissues.

The enzyme from domestic fowl liver has a K_m for glucose-6-phosphate of 3-8 mM and its activity in chick embryos increases to maximum just before hatching. Glucose-6-phosphatase activity in turkey liver is related to the carbohydrate content of the diet, e.g. it is increased 4-fold on fasting compared with levels on a diet containing 55% carbohydrate (Donaldson and Christensen, 1991). A number of studies have been made on the efficacy of various metabolites as gluconeogenic precursors in avian liver (Stevens L., 2004). There are some variation in the rate of gluconeogenesis obtained, depending on the type of preparation used, but the order is typically: lactate ≈ glycerol > pyruvate > alanine > aspartate > serine (Langslow, 1978). In avian liver, lactate is the best substrate, in the mammalian liver pyruvate is better than lactate.

Birds appear capable of higher rates of gluconeogenesis than mammals and carnivorous birds are generally able to carry out higher rates of gluconeogenesis than graminiferous birds. In a study comparing the black vulture and domestic fowl when fasted, it was interesting to observe that domestic fowl synthesized higher level of PEPCK, glucose-6-phosphatase, alanine amino transferase, aspartate amino transferase in the liver, in order to increase the capacity for gluconeogenesis, where very little change occurred in the case of black vulture, presumably because it already had sufficient capacity. Most of the detailed studies on gluconeogenesis in birds have been carried out using either the liver and the kidney of domestic fowl or pigeon, but the Japanese quail has also been studied although to a more limited extend. It is clear from some studies that although the pathways used very similar to those used by mammals there are differences in their relative capacities and their control. Both liver and kidney are capable of high rates of gluconeogenesis. In avian kidney, gluconeogenesis occurs predominantly in the cortex (Yorita *et al*., 1987) and in liver it occurs in the parenchyma cells. In the domestic fowl, approximately 70% of gluconeogenesis is occurred in the liver and the remainder in the kidney. The main glycogen reserves in birds are

present in the liver, kidney and skeletal muscle. However, in the skeletal muscle, it is metabolized anaerobically to lactate. Lactate is released into the bloodstream, where it is mainly taken up by the liver and used for gluconeogenesis.

Pancreas and Insulin-glucagon

Generally, the pancreas of vertebrates was divided into two parts, exocrine and islets of Langerhans. Endocrine cells are generally concentrated in the islets of Langerhans although some endocrine cells which were found in islets of Langerhans were also dispersed between the exocrine acini and epithelial lining of secretory ducts, especially pancreatic polypeptide and somatostatin cells which were numerous in that region (Iwanaga *et al*., 1983; Alumets *et al*., 1977). The islets, which are made up of different proportions of these cell types, are classified as dark or light islets. The predominant cell type is the darker islets is A (glucagon secreting), but with some B, D and PP cells, whereas the lighter islets have B (insulin) cells as the predominant type. Morphologically, the avian pancreas, a tongue-shaped structure has three distinct lobes; splenic, dorsal and ventral (Clara *et al.,* 1924). But in 1962, Mikami and Ono reported that the ventral lobe has a longitudinal cleft running throughout its length giving the appearance of two separate lobes. The central portion of this, closet to the dorsal lobe, is sometimes referred to as the third lobe. Ono (1967) insisted that differentiation of the pancreatic lobes occurs after 10 days of incubation.

Foltzer *et al.,* (1987) estimate that 80% of the glucagon and 63% of the somatostatin are concentrated in the splenic lobes of the duck and 53% of the insulin is concentrated in the dorsal lobe. The higher concentration of glucagon in the splenic lobe of the ostrich pancreas suggests the cell distribution in this species is similar to that of the domestic fowl and quail (Ferreira *et al*., 1991). Type A cells are generally more abundant in avian species that in other vertibrates (Guha and Ghosh, 1978). Until now, abundant researches about avian endocrine pancreas were reported, including electron microscopical studies and histochemical studies using silver techniques (Mikami and Mutoh, 1971; Smith, 1973). Many researchers have

shown great concern for the anatomical, histological and endocrinological structure of the splenic lobes. Recently, immunohistochemistry using the specific antisera against hormone was settled (Sternberger, 1979). Existence, distribution and relative frequency of various hormones producing cells including insulin, glucagon, somatostatin, pancreatic polypeptide, biotin, serotonin and chromogranin-immunoreactive cells were demonstrated in the pancreas of avian species including chicken (Kalliecharan and Steeves, 1982; Bagnell *et al*., 1989; Cooper *et al*., 1997), duck (Lucini *et al*., 1996) and mallard (Lee *et al*., 1998a; 1998b). In addition, some fragmental reports (Alumets *et al*., 1977; 1978; Bagnell *et al*., 1989) about ontogeny of immunoreactive cells in the avian pancreas were reported.

Glucagon is the dominant pancreatic hormone in birds compared with mammals but very few reports are available on glucagon concentration in chick embryos and hatched chicks. Glucagon helps to maintain the glucose requirement of chicken embryo during embryogenesis and has an important role for transition from high-fat, low carbohydrate diet to low fat-high carbohydrate diet in hatched chick (Langslow *et al*., 1979). On the other hand, insulin regulates the concentrations of amino acids and related compounds in plasma, amniotic fluid, and allantoic fluid of 13-d-old chicken embryos (Hohlweg *et al*., 1999) and accelerate chick embryonic growth and morphological development also (de Pablo *et al*., 1991). So, glucagon is known as a “hormone of fasting” and insulin as a “hormone of feasting” (Hazelwood, 2000).

Chapter 2

Ontogenic profile of glucokinase and hexokinase mRNA expressions in embryonic chicken liver and muscle

Introduction

Glucose, the primary source of energy, is transported into the cell from the blood by glucose transporters (GLUT) and thereafter catalyzed into glucose-6-phosphate by hexokinases (HK; EC 2.7.1.1), which makes glucose available for further utilization. This catalytic reaction has a large negative free energy change (ΔG = 16.7 kJ/mol) and is an important control point of glycolysis and glycogenesis. In mammals, four hexokinase isoenzymes (HKI, HKII, HKIII and HKIV, otherwise known as glucokinase, GK; EC 2.7.1.2) have been characterized (Kanno, 2000). With the help of GK, mammalian liver plays a central role in glucose homeostasis by balancing the uptake and release of glucose (Postic *et al.*, 2004, Stalmans, 1976). The mammalian muscle also plays a major role in the clearance of blood glucose in the postprandial state (DeFronzo *et al.,* 1992). In the rat skeletal muscle HKII and, to a lesser extent, HKI are the major isoforms of hexokinase (Postic *et al.,* 1994).

Among hexokinase isoenzymes found in chicken, HKI and HKII have been cloned and sequenced (Seki *et al.,* 2005), and their gene expressions have been identified in various tissues including the skeletal muscle, brain, heart, liver, kidney, testis and ovarian follicle as well as lymphocyte (Seki *et al.,* 2005; Seol *et al.,* 2006; Shashidhara *et al.,* 2007). Partial cDNA cloning, immunodetection and enzymatic activity of chicken GK have been done (Berradi *et al.,* 2005) and changes in its mRNA, protein and enzymatic activity in the liver in response to dietary carbohydrates have also been investigated (Rideau *et al.,* 2008). However,

a little (less) information is available so far concerning the developmental changes of HK isoenzymes. This study was, therefore, undertaken to clarify mRNA expression profiles of HK isoenzymes in the liver and skeletal muscle of chicken embryos to elucidate their roles in maintaining high plasma glucose during embryogenesis.

Materials and methods

Animals and sampling

The fertile eggs of white leghorn chicken from GHEN Corporation (Gifu, Japan) were incubated under 70% humidity and 37.8ºC. E0 was defined as the start day of incubation. Livers, kidneys and muscles from chicken embryo of E13 to E21 were collected, washed with PBS slightly, snap frozen in liquid nitrogen. Blood was also collected with 1 ml injection syringe (Terumo Ltd., Tokoyo, Japan) and centrifuge at 12000×g. Tissues and blood samples were stored at –80ºC until further processing. All the experiments were performed in accordance with Regulation for Animal Experiments in Gifu University.

RNA isolation and reverse transcription

Using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) total RNA was extracted from frozen livers, kidney and muscles. Firstly the tissues were taken in the eppendorf tube which was already filled with 1 ml of Trizol reagent then the tissues were sonicated. After homogenization the samples was incubated for 5 minutes at room temperature and 0.2 ml chloroform for per ml of Trizol was added in the sample. Then the tube was vigorously shacked by hands for 15 seconds and the tube was incubated at room temperature for 2-3 minutes. Finally after centrifugation at 12000×g for 15 seconds at 4ºC, 400 μl aqueous phase was collected.

After transferring the aqueous phase to a fresh tube 0.35 ml isopropanol was added and the sample was centrifuged at 12000×g for 10 minutes at 4ºC. The supernatant was removed and RNA pellet was washed once with 1 ml of 75% ethanol. Then the RNA pellet was centrifuged at 7500×g at room temperature. The RNA pellet 5-10 minutes was dried and was dissolved RNA in RNase-free water.

By using Nanovue spectrophotometer (GE Healthcare, Little Chalfont, Buckinghamshire,

UK) the purity and concentration of the isolated RNA were assessed. 1 μg of total RNA was reverse transcribed into first strand cDNA by using ReverTraAce (TOYOBO CO. LTD, Osaka, Japan). Firstly the RNA solution was prepared by dilution and made it into 1 μg/μl concentration. The premix was prepared with the 5×RT buffer, random primer (25 pmol/μl), 10 mM dNTPs, and ReverTraAce according to the guideline from manufacturer. The premix was taken into some PCR tube and 1 μl of total RNA was added. And finally the PCR tube was incubated at 30ºC for 10 minutes and 42ºC for 60 minutes and inactivated the PCR tube for 5 min at 99ºC.

Sequencing

cDNA was amplified by PCR using gene specific primers (Table 1), designed from previously reported *Gallus gallus* HKI, HKII and GK sequences (accession numbers NM_204101.1, NM_204212.1 and AF525739.4, respectively) using Primer3 software (http://frodo.wi.mit.edu/) and KOD -plus- DNA polymerase (TOYOBO). The PCR products and RACE (rapid amplification of cDNA ends) products were electrophoresed in 1.5% agarose gel and purified with Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, USA). For purification the gel was sliced with a razor blade above and below the bands of interest and put it in the 1.5 ml microcentrifuge tube. Before and after taking the gel into the tube, the weight of tube was recorded. Membrane binding solution (MBS) was added at a ratio of 1 μg: 1 μl MBS. After vortex and incubation (at 60ºC for 10 minutes) the dissolved gel mixture was transferred to SV minicluomn (Promega corporation) assembly and incubated for 1 minute at room temperature. The content was discarded after centrifugation (16000×g for 1 minute). To wash the column, 700 microliter membrane wash solution (MWS) which was mixed with ethanol was added to the SV minicolumn and it was centrifuged (16000×g for 1 minute). Then again after centrifugation (16000×g for 5 minutes) and washing the column with MWS, the nuclease free water was directly added to the center

of the column (the column was put into a autoclaved 1.5 ml microcentrifuge tube) and incubated at room temperature for 1 minute. Finally after centrifugation (16000×g for 1 minute), the microcentrifuge tube was preserved at 4ºC. Purified products were sequenced with an ABI automated sequencer (Applied Biosystems, Courtabouef, Freance). The resulting sequences were blasted with known sequences from GenBank database using the basic alignment search tool (blast.ncbi.nlm.gov).

Table 1. **Primers used for sequencing**

Primer	Sequence (5'→3')	Amplicon (bp)	Accession number
HKI-Seq1	Forward CAGGACAGAGTTTGATAGAG Reverse CAGGTAACGATGTGGTC	924	NM_204101.1
HKI-Seq2	Forward AACGGTTGAAATGCACAACA Reverse GCTCACCATCCACCATCTCT	446	NM_204101.1
HKI-Seq3	Forward ATGTGGTAGCTGTAGTGAAC Reverse AGGTCTTTAGGAGAAGGTG	860	NM_204101.1
HKII-Seq1	Forward GGACAAATACCTGTACCAC Reverse CTTCTCAATAGCGGAGAC	967	NM_204212.1
HKII-Seq2	Forward GAGTTCGACCATGAGATAG Reverse GGAAGGAGAAGGTGAAG	988	NM_204212.1
HKII-Seq3	Forward GAGATTTCCTCGCTCTG Reverse GTGGGTGCAGTTTGTAG	1032	NM_204212.1
GK-Seq1	Forward ATTTCCTCTCGCTGGATCTG Reverse CCAAATGCGCCCCACTCCGT	565	AF525739.4

Determination of cycle number within linear phase

Before semi-quantification, a range of PCR cycle numbers showing linear amplification was determined for each gene by running PCR with cDNA samples from each day of incubation and measuring band intensities by agarose gel electrophoresis between 11 and 35 PCR cycles. PCR cycle numbers determined as such were 29 for muscle HKI, 27 for liver HKI and muscle HKII, 29 for liver HKII, 34 for muscle and liver GK and 12 for muscle and liver 18s rRNA (a house keeping gene).

Table 2. **Primers used for semi-quantitative PCR**

Primer	Sequence (5'→3')	Amplicon (bp)	Accession number
HKI-A	Forward CTGCCAAAGTGAAAATGCTG	305	NM_204101.1
	Reverse CTTGCAAGGGAAGGAGAATG		
HKII-A	Forward GAAGTCGGGCTGATAGTTGG	432	NM_204212.1
	Reverse GCTGTCATCACACGTGCTCT		
GK-A	Forward ATTTCCTCTCGCTGGATCTG	382	AF525739.4
	Reverse ACCACGTCCATCTCGAAGTC		

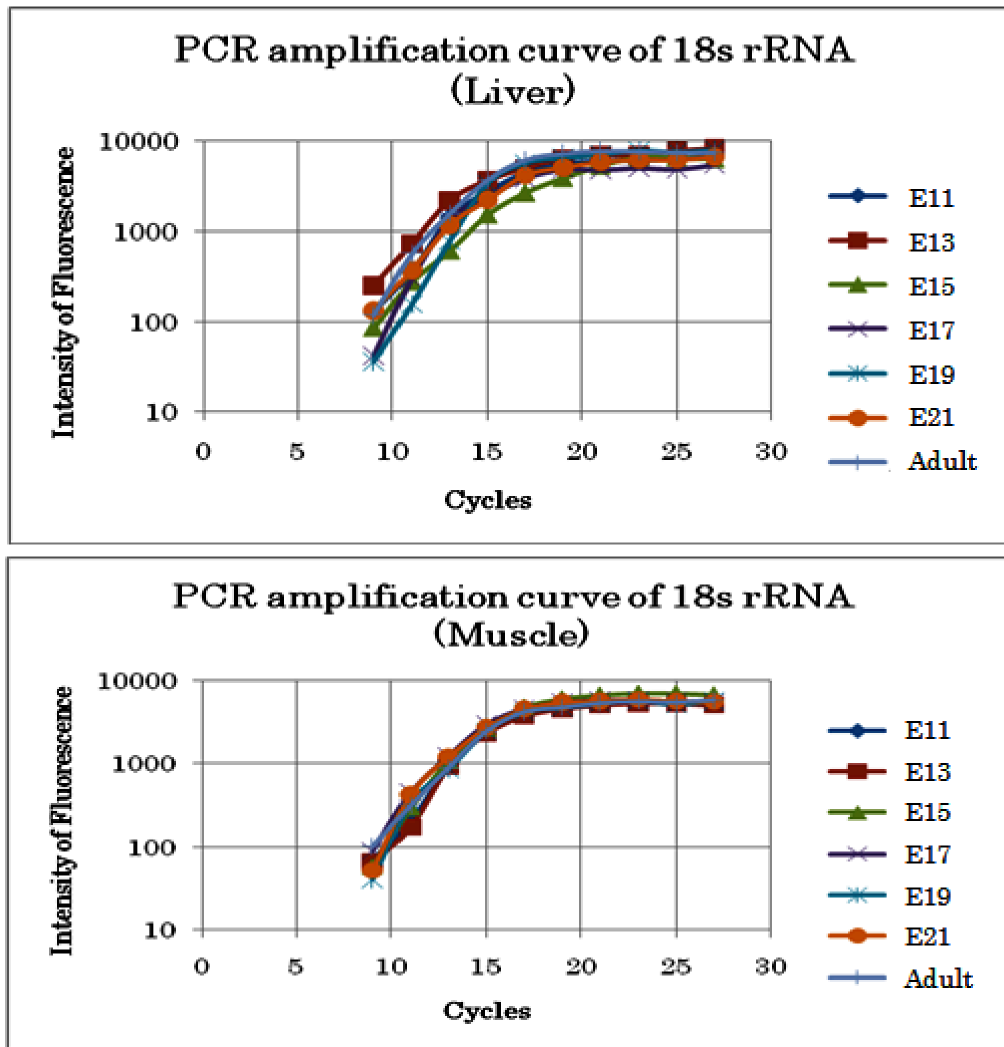


Fig 1. **PCR amplification curve for 18s rRNA.** First strand cDNA was made by random priming from 1 µg of total RNA and then 500 ng cDNA were amplified by gene specific primers. PCR was performed from 9 cycles to 27 cycles. After PCR amplification 10 µl of PCR product was electrophoresed on 2% of agarose gel in 0.5×TBE buffer. Fluorescence of each band was measured by Image J software. Values are mean ± S.E.M. for 3-4 chicken embryos.

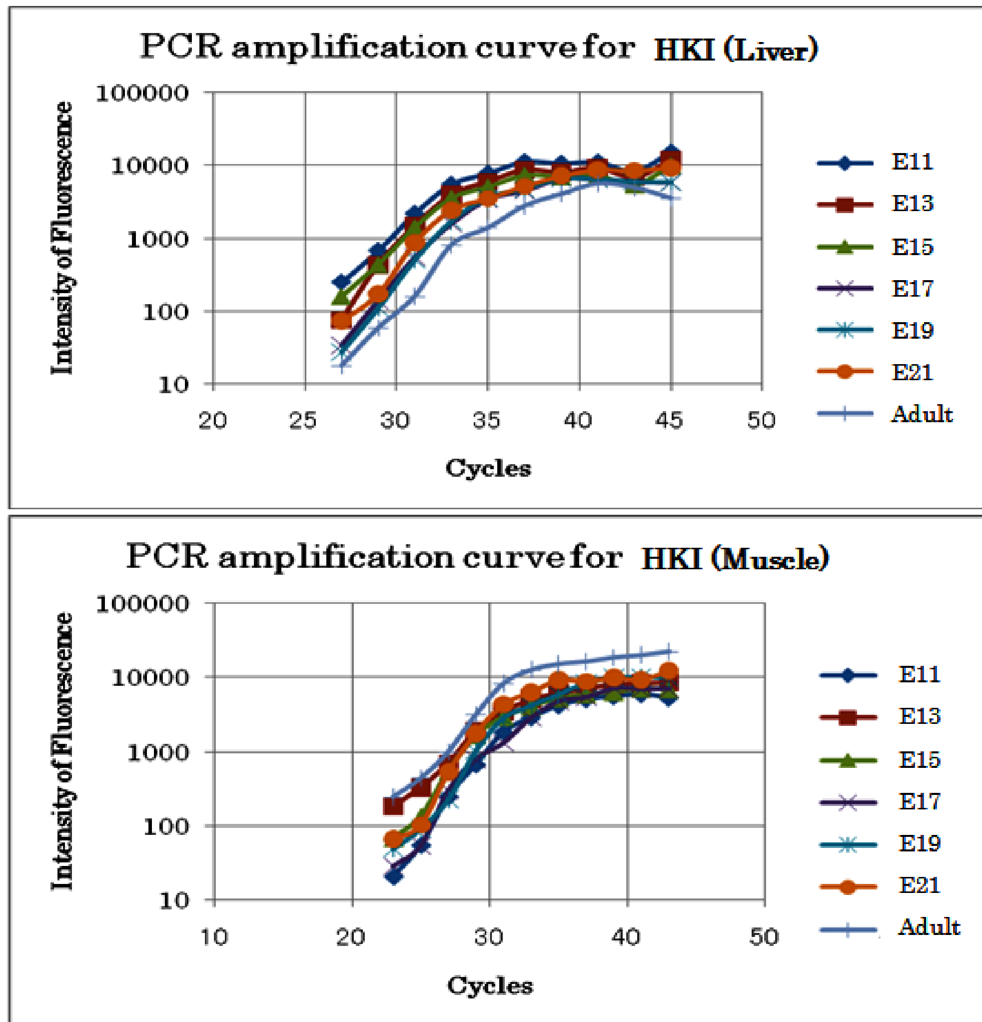


Fig 2. **PCR amplification curve for HKI.** First strand cDNA was made by random priming from 1 μg of total RNA and then 500 ng cDNA were amplified by gene specific primers. PCR was performed from 23 cycles to 45 cycles. After PCR amplification 10 μl of PCR product was electrophoresed on 2% of agarose gel in 0.5×TBE buffer. Fluorescence of each band was measured by Image J software. Values are mean ± S.E.M. for 3-4 chicken embryos.

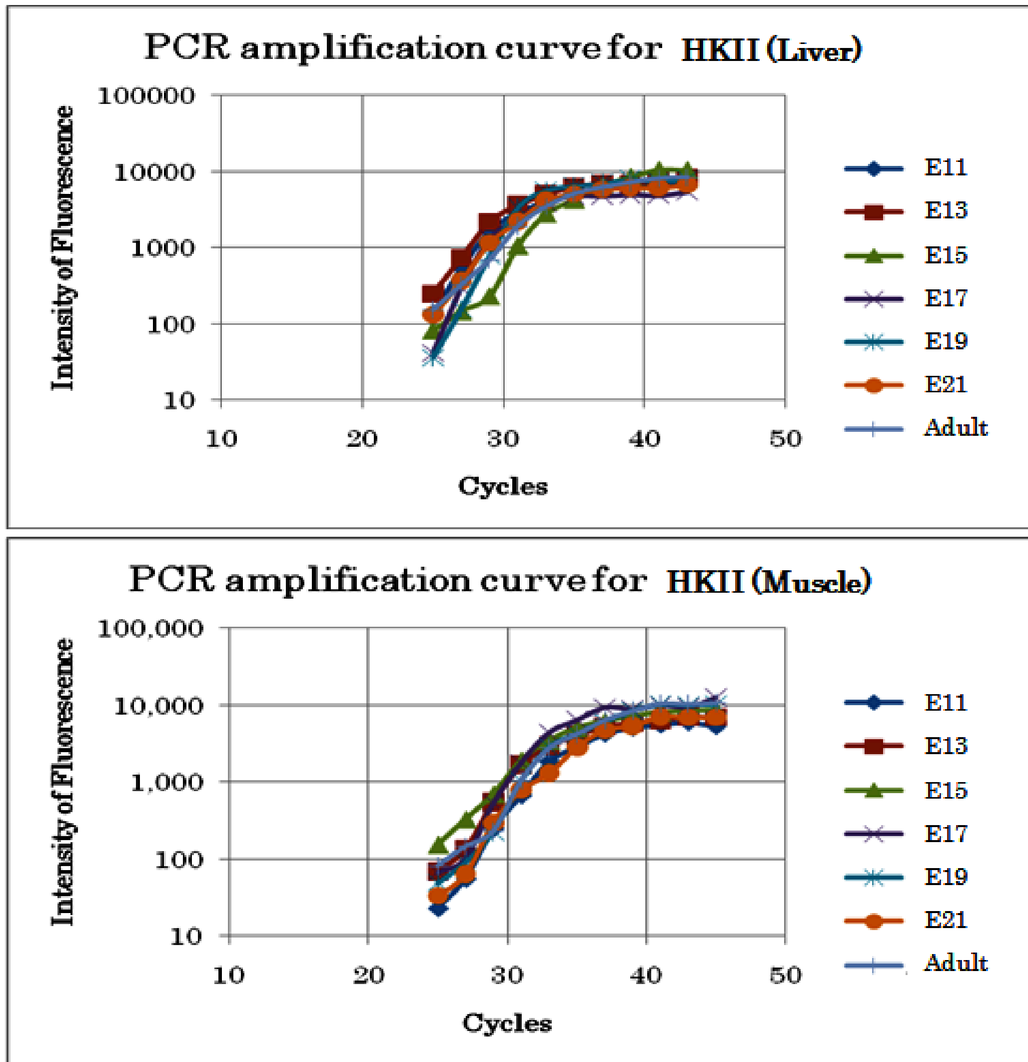


Fig 3. **PCR amplification curve for HKII.** First strand cDNA was made by random priming from 1 µg of total RNA and then 500 ng cDNA were amplified by gene specific primers. PCR was performed from 25 cycles to 45 cycles. After PCR amplification 10 µl of PCR product was electrophoresed on 2% of agarose gel in 0.5×TBE buffer. Fluorescence of each band was measured by Image J software. Values are mean ± S.E.M. for 3-4 chicken embryos.

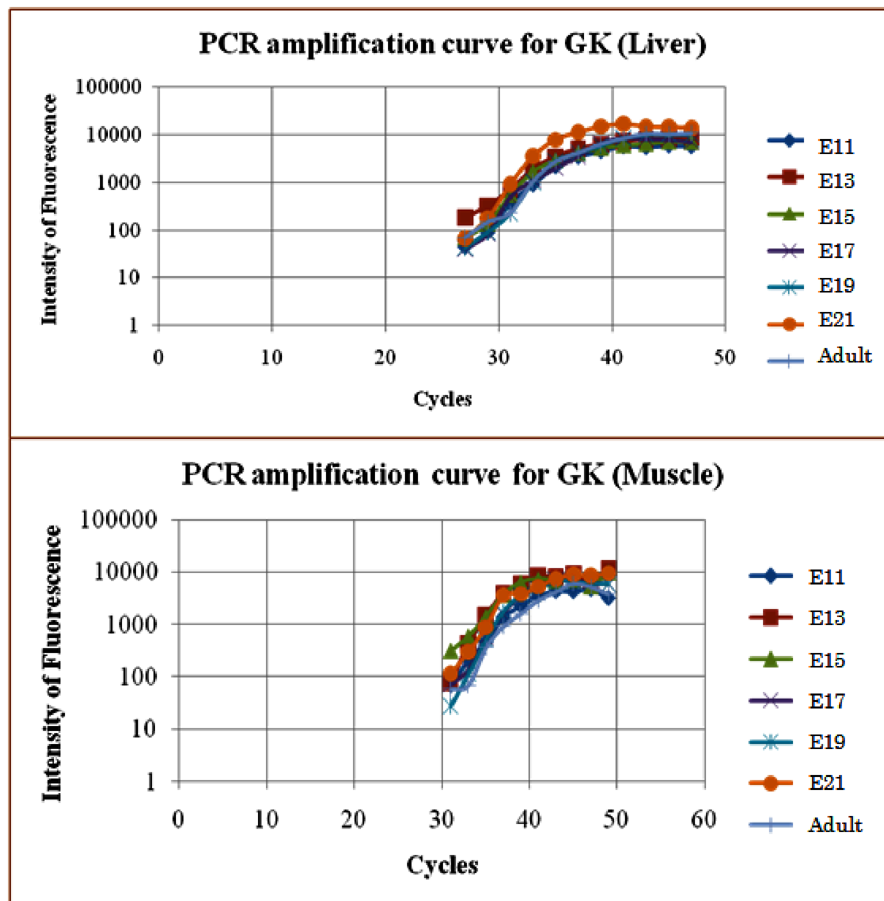


Fig 4. **PCR amplification curve for GK.** First strand cDNA was made by random priming from 1 µg of total RNA and then 500 ng cDNA were amplified by gene specific primers. PCR was performed from 27 cycles to 49 cycles. After PCR amplification 10 µl of PCR product was electrophoresed on 2% of agarose gel in 0.5×TBE buffer. Fluorescence of each band was measured by Image J software. Values are mean ± S.E.M. for 3-4 chicken embryos.

Semi quantification by PCR and Gel electrophoresis

Each gene expression was measured semi-quantitatively by PCR using gene specific primers (Table 2) that were designed from the determined sequences. Semi-quantitative PCR was carried out in a total volume of 25 µl including 1X buffer for KOD –plus-, 0.2 mM dNTPs, 1 unit KOD –plus-, 0.3 µM forward and reverse primers and 500 ng cDNA.

Concentration of $MgSO_4$ was 1.1 mM for HKI, 1.2 mM for HKII and GK, 1 mM for 18s rRNA. Amplification of cDNA was achieved with an initial denaturation at 94ºC for 2 min followed by denaturation (94ºC for 30 sec), annealing (30 sec at 51.2ºC for HKI and 55.4ºC for HKII, 54.3ºC for GK, 55ºC for 18s rRNA), and extension (68ºC for 1 min). For every isoenzyme, negative controls (autoclaved water) were included to ensure that observed bands were not simply from contamination. After defined cycles of PCR, 10 µl of the amplified product was electrophoresed on a 2% agarose gel in 0.5X TBE buffer and thereafter the gel was stained with ethidium bromide. Intensity of the ethidium bromide fluorescence of each band was measured by the Image J Imaging System (http://rsbweb.nih.gov). To establish the size of the PCR product and to normalize the condition of gel staining and Image J measurement, a constant amount of molecular weight marker (100 bp DNA Ladder; New England Biolabs, Ipswich, MA, USA) was electrophoresed in 2 lanes every time.

Restriction digestion

To confirm gene specific PCR amplification for semi-quantification, restriction enzymes *Apa*LI (New England Biolabs), *Rsa*I (Promega) and *Eag*I (Fermentas, Burlington, Ontario, Canada) were used to digest HKI, HKII and GK PCR products. These enzymes were selected so that they could yield fragments with the lengths specific to each gene. Digested samples were electrophoresed on 2% agarose gel and the lengths of the restriction fragments were estimated with Gene Ladder 100 (Wako Nippon Gene, Tokyo, Japan).

Statistics

Data are presented as means ± SEM. Comparisons among groups were performed with one way ANOVA followed by Tukey's multiple comparison test using SPSS statistical package (ver. 13.00). Differences were considered statistically significant at $P < 0.05$

Results

Sequence analysis of HKI, HKII and GK

Total RNAs extracted from the liver and the skeletal muscles were reverse-transcribed and amplified with gene specific PCR primers listed in Table 1, followed by sequencing. It was observed that both organs gave amplicons specific for HKI, HKII and GK, and their sequences were about 99% identical with the sequences of chicken HKI, HKII and GK, respectively, available in the GenBank database (Table 3).

Table 3. **Sequence analysis of HKI, HKII and GK**

Enzyme	Organ	Length (bp)	Blasted with	Identity
HKI	Muscle and liver	1915	*Gallus gallus* HKI (NM_204101.1)	99%
HKII	Muscle and liver	2338	*Gallus gallus* HKII (NM_204212.1)	99%
GK	Muscle and liver	565	*Gallus gallus* GK (AF525739.4)	98%

Specificity of amplified product

Both the liver and the skeletal muscle expressed three hexokinase isoenzymes and, the restriction enzymes *Apa*LI, *Rsa*I and *Eag*I also digested HKI, HKII and GK PCR products consecutively in both organs, yielding fragments with lengths specific to each gene.

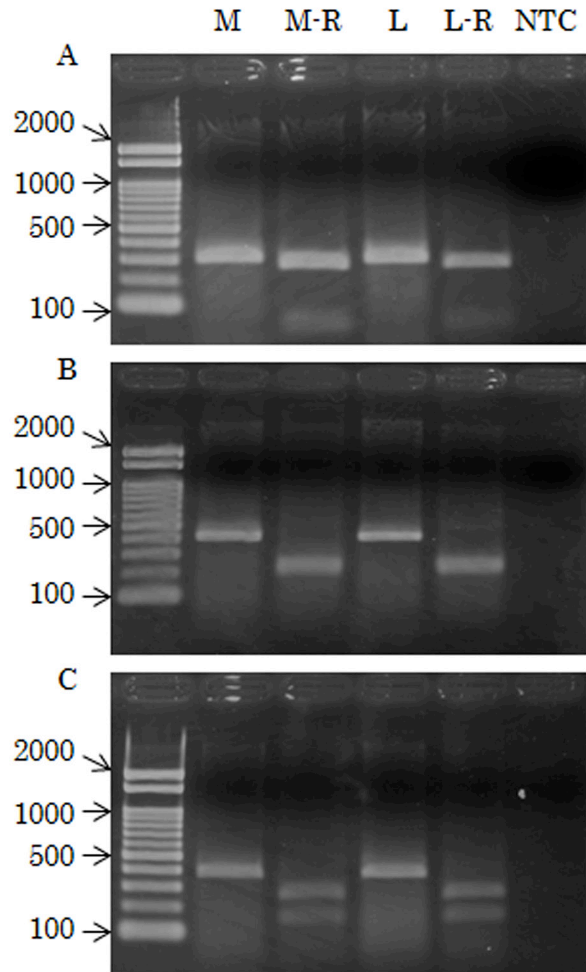


Fig 5. **Specificity of PCR products amplified with primers for HKI (A), HKII (B) and GK (C).** Total RNA extracted from the skeletal muscle and the liver was reverse transcribed and amplified with gene specific primers listed in Table 2, followed by restriction enzyme digestions. Specific amplicon of HKI (305 bp) yields 43 and 262 bp restriction fragments only when it is digested with *Apa*LI. Likewise, amplicon of HKII (432 bp) yields 203 and 229 bp fragments with *Rsa*I; amplicon of GK (382 bp) yields 136 and 246 bp fragments with *Eag*I. M: amplicon from the skeletal muscle, M-R: restriction digested product of M, L: amplicon from the liver, L-R: restriction digested product of L, NTC: non-template control.

Semi-quantitative mRNA expression analysis of hexokinase isoenzymes

In the liver, HKI mRNA expression decreased on E17 and remained decreased until E21. HKII mRNA expression increased from E11 to E13 and remained increased thereafter. GK mRNA expression gradually increased from E11 to E21. In the skeletal muscle, HKI increased on E13 and remained high thereafter. HKII mRNA expression showed a tendency similar to HKI, although the changes were not significant. GK mRNA expression significantly decreased on E19 and remained decreased on E21.

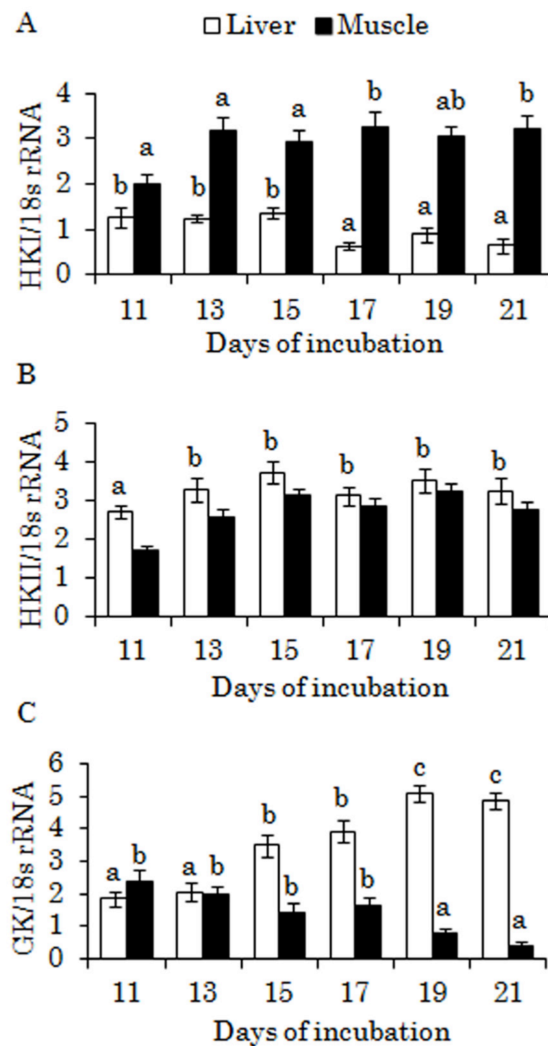


Fig 6. **Changes in mRNA levels of hexokinase I (HKI; panel A), hexokinase II (HKII; panel B) and glucokinase (GK; panel C) in the liver and the skeletal muscle during chicken embryogenesis**. First strand cDNA was made by random priming from 1 µg of total RNA and then 500 ng cDNA were amplified by gene specific primers. After PCR amplification 10 µl of PCR product was electrophoresed on 2% of agarose gel in 0.5×TBE buffer. Fluorescence of each band was measured by Image J software. Values are mean ± S.E.M. for 5-6 chicken embryos. Different alphabets show significant difference within the same organ (liver or muscle) by Tukey's multiple range test ($p<0.05$).

Discussion

In my study with white leghorn chicken embryos, HKI was expressed in the liver and GK in the skeletal muscle, although they both exhibited significant decrease toward the end of incubation (Figs. 6A and C). In both liver and skeletal muscle, HKII mRNA was expressed during the experimental period (Fig. 6B). These results are not consistent with the previous reports (Berradi *et al.,* 2005; Seki *et al.,* 2005) where neither HKI was found in the liver nor GK in the skeletal muscle. Seki *et al*., (2005) also reported that HKII mRNA was highly expressed in the skeletal muscle but very low in the liver. However, these reports differ from my study in that they used adult broiler chicken. In rats, expression profiles of the hexokinase isoenzymes differ between times of ontogenesis. Expression of GK mRNA, protein and enzyme activity become detectable in the liver after weaning (García-Flores *et al.,* 2002; Iynedjian *et al.,* 1987; Postic *et al.,* 1994; Walker and Holland, 1965). The amount of mRNA and enzyme activity of HKI in the skeletal muscle and liver become decrease after birth, whereas HKII in the skeletal muscle become expressed concomitantly with weaning (Postic *et al*., 1994). I thus speculate that expression profiles of HK isoenzymes also differ between embryonic and non-embryonic chickens.

The glucose concentration at which chicken hepatic GK is half-saturated is about 8 mM (Berradi *et al.,* 2005; Borrebaek *et al.,* 2007). Since the blood glucose level of adult chicken is about 11 mM (200 mg/dl), the possible fluctuations of plasma glucose concentrations could be reflected directly to the rate of glucose phosphorylation in the hepatic cells, thus probably providing the liver an important characteristic in maintaining the blood glucose homeostasis that is well-established in mammals (Postic *et al.,* 2004; Stalmans, 1976).

The present results that the expression of GK mRNA in the liver increases as the developmental stage proceeds (Fig. 6C) may suggest that chicken liver acquires its role in blood glucose homeostasis gradually during the embryogenesis but neither suddenly after hatch nor with the start of feeding. This is supported by the work of Borrebaek *et al.* (2007)

who reported that the enzyme activities of hepatic GK and HK of white leghorn chicken embryo increased before hatching. In addition, my finding of GK mRNA expressed in the skeletal muscle, especially, in the earlier stages of the experimental period (Fig. 6C) may suggest the possibility that the skeletal muscle plays a liver-like role in glucose homeostasis in some stages of development. This possibility should be carefully tested, by determining protein level and enzymatic activity of GK in the embryonic skeletal muscle.

It is reported that glycogen contents of chicken embryos rapidly increase from E12-13 in the liver (Ballard and oliver, 1963; García *et al*., 1985) and gradually increase from E10 in the skeletal muscle (García *et al*., 1985). Thus, increased expression of HKII and GK in the liver (after E11) and HKI and HKII in the skeletal muscle (on E13) (Fig. 6) could contribute to an increase of glucose uptake from the blood into these organs that may lead, to some extent, to increased glycogen contents, thereby playing roles in glucose homeostasis of chicken embryos.

Finally, the present study showed expression of GK in the muscle and HKI in the liver of chicken embryos. Further studies are required to understand whether these transcripts produce protein with physiological role.

Chapter 3

Ontogenic profile of gluconeogenic key enzymes mRNA expressions in embryonic chicken liver, kidney and muscle

Introduction

It has been 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 expression as well as transcriptional and post-transcriptional processes would help to better understand how the enzyme actually working *in vivo*. Thus it was necessary 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 (Steven, 1996) between day 13 and day 21 of chicken embryos in liver, muscle and kidney. Since the reactions catalyzed by these four enzymes are virtually irreversible, they are the rate-limiting enzymes of gluconeogenesis (Granner and Pilkis, 1990; Stevens, 1996; Jitrapakdee *et al*., 2008). mRNA expression of all four key enzymes in liver, kidney and muscle suggested a possibility of active gluconeogenesis in these tissues that could lead to high plasma glucose in chicken embryo.

Materials and methods

Animals and sampling

The fertile eggs of white leghorn chicken from GHEN Corporation were incubated under 70% humidity and 37.8ºC. E0 was defined as the start day of incubation. Livers, kidneys and muscles from chicken embryo of E13 to E21 were collected, washed with PBS slightly, snap frozen in liquid nitrogen. Blood was also collected with 1 ml injection syringe (Terumo Ltd., Tokoyo, Japan) and plasma was isolated with 0.5M EDTA and centrifugation at 15000×g. Tissues and blood samples were stored at –80ºC until further processing. All the experiments were performed in accordance with Regulation for Animal Experiments in Gifu University.

Quantification of blood glucose

The plasma glucose concentrations were measured by glucose oxidase (GOD-PAP) method using Glucose CII (Wako Pure Chemical Co., Ltd. Osaka, Japan) as recommended by the manufacturer.

RNA isolation and reverse transcription

This procedure was performed according to the protocol described in chapter II.

Sequencing

cDNA sequence was determined by using conventional PCR, and 5' RACE. For conventional PCR cDNA samples were amplified by using 2 μM of designed primers for sequencing (Table 1) with 1 X buffer for KOD -plus-, 0.2 mM dNTPs, 1 unit KOD -plus- DNA polymerase, 25 mM Mg, and 500 ng cDNA. The reaction volume was 50 μL. Amplification of cDNA was achieved with an initial denaturation at 94ºC for 2 minutes followed by 35 cycles of denaturation (94ºC for 30 sec), annealing 30 sec at different

temperature depend on primer sets (Table 1) followed by extension (68ºC for 1 min). 5' end of FBPase was amplified by using 5'RACE system for rapid amplification cDNA ends (Invitrogen). The PCR products and RACE products were electrophoresed in 1.5% agarose gel and purified with Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, USA). For purification the protocol of chapter II was followed.

Table 1. **Primers used for sequencing**

Enzyme	Primer	Sequence (5'→3')	Accession no	T_a (ºC)
mFBPase	mFBPase-1 1	TGCTGGGTATCTCAACAC AGCATGAAGCAGTCCAC	XM_425039.2	51
	mFBPase-1 2	AGGAGACAGATGATGAGC TCCCTGTAGTTGCTATCC		53
	mFBPase-1 3	ATATGGTGCCAGGTATG CTGAGAAGTGGGTGTTG		49
lFBPase	lFBPase-1 1	CTTCGTGATGGAAGAGG TCCTGAGATACTCGGTGAC	XM_425040.2	51
	lFBPase-1 2	AATGCTGGTACTGGCTAC TCTGGCTGTTCAGACTTG		51
	lFBPase-1 3	CTGATCTCCCGTCACATTGG CGCATGTGCTGAAAGATGAC		54
G6Pase	G6Pase 1	TTCCAGTGCTAACAGACTC AACAGACCACAGCAAGTC	XM_422017.2	51
	G6Pase2	GCTATTCAGACAGCAAGC CTACTTACCAGGAGCACATC		53

T_a – Annealing temperature

Real Time PCR analysis

The primers (Table 2) for real-time PCR were designed with oligo perfect software (www.invitrogen.com/oligos.I). Real time PCR amplification of gluconeogenic key enzymes was performed on the ABI prism 7000 apparatus (Applied Biosystems) using SYBR Premix Ex Taq II (Perfect Real Time) (Takara, Otsu, Shiga, Japan). 2 μL of reverse transcribed product were added to a 20 μL mix containing 10 μL SYBR Premix Ex Taq II (2X), 0.4 μM of each primer, 0.4 μL of 50X ROX reference dye, and 6 μL water. Cycling conditions were 40 cycles of 95ºC for 5 sec, 60ºC for 20 sec and 72ºC for 31 sec after initial 30 sec incubation at 95ºC. The reference was 18s rRNA. Dissociation curve for every gene was performed at the end of the PCR by slowly heating samples from 60 to 95ºC and continuous recording of SYBR Green fluorescence was also done to assess the specificity of the amplification of. For each sample the threshold (C_t) was determined. Standard curves for each primer pair were made from serial dilutions of a cDNA pool and PCR efficiencies (E) were calculated according the equation $E = 10^{(-1/slope)}$ by performing that standard curves. The relative quantities of gluconeogenic key enzymes were calculated by standard curve method.

Statistics

Data are presented as means ± SEM. Comparisons among groups were performed with one way ANOVA followed by Tukey’s multiple comparison test using SPSS statistical package (ver. 13.00). Differences were considered statistically significant at $P < 0.05$

Table 2. **Primers used for real-time PCR**

Enzyme	Primer sequence (5'→3')	Length (bp)	Accession no
PC	TGGGGCATAAATTCAAGGAG GAATTCGACCACGGAGAGAG	194	AF509529.1
PEPCK-C	TGCTGGTGTGCCTCTTGTAT CACACGGGAATTCTCTCCAT	295	M14229.1
PEPCK-M	GTTACCTGGAACATTGGCTGTC CAGGTCCAAATCCCCTTCTT	224	J05419.1
mFBPase	AACTACAGGGACAGAGGCG AAGTGGGTGTTGAAATGCTA	228	XM_425039.2
lFBPase	CGGGAGATCAGGTGAAGAAA ATGGTCCCAATGGAAACAAG	208	XM_425040.2
G6Pase	CATGTACTTCACTTACTTTCCTCC TATTGTATCAGCGTGGCGTA	137	XM_422017.2
18s rRNA	TCAACTTTCGATGGTACTGTCTGTG CTTGGATGTGGTAGCCGTTTCT	106	AF173612.1

Results

Plasma glucose

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).

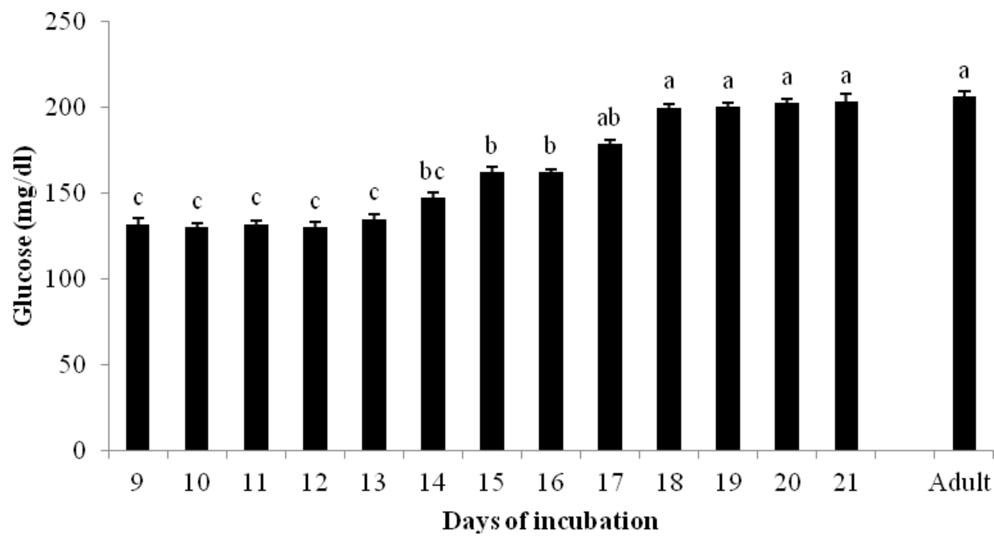


Fig 1. **Developmental changes of plasma glucose in chicken embryo (mg/dL).** Different letters indicate significant differences among days of incubation ($P<0.05$). Values are means ± SEM; n = 10.

Sequencing

The determined partial cDNA sequences of mFBPase, lFBPase, and G6Pase from muscle were aligned with sequences from GeneBank database XM_425039.2, XM_425040.2, and XM_422017.2 respectively. The sequences were 99.56%, 100% and 100% similar with database (Table 3).

Table 3. **Sequence analysis of G6Pase, lFBPase and mFBPase**

Enzyme	Length (bp)	Blasted with	Identity
mFBPase	1135	*Gallus gallus* FBPase (XM_425039.2)	99.56%
lFBPase	900	*Gallus gallus* FBPase (XM_425040.2)	100%
G6Pase	1093	*Gallus gallus* G6Pase (XM_422017.2)	100%

Real time PCR data analysis

Figure 2 shows relative gene expressions of the gluconeogenic key enzymes in liver, kidney and skeletal muscle. In liver the expression of all gluconeogenic key enzymes mRNA were appeared from first day of my experiment, E13. And PC, PEPCK-C, PEPCK-M and G6Pase attain their utmost expression on E15 whereas lFBPase and mFBPase showed their maximum expression on E13. However, mRNA expression of all enzymes were started to decline immediately after reaching its highest level and showed their lowest level on E19 or E21 except G6Pase. G6Pase maintained its highest level up to day E19 and then sharply decreased to its lowest level.

In kidney (Fig 2), PEPCK-C, mFBPase and lFBPase showed their maximum expression on E13, whereas PC, PEPCK-M and G6Pase demonstrated their utmost expression on E15. After E15 all the enzymes showed declining tendency except PEPCK-C. G6Pase was increased again on E21.

G6Pase and mFBPase, only these two key enzymes of gluconeogenesis were expressed in

muscle from early of E13 of incubation. On E15 G6Pase and mFBPase demonstrated opposite fashion. More interestingly, after E15, G6Pase had a decreasing tendency and reached its minimum level on E21. On the other hand, mFBPase had an increasing tendency and reached its maximum level on E19.

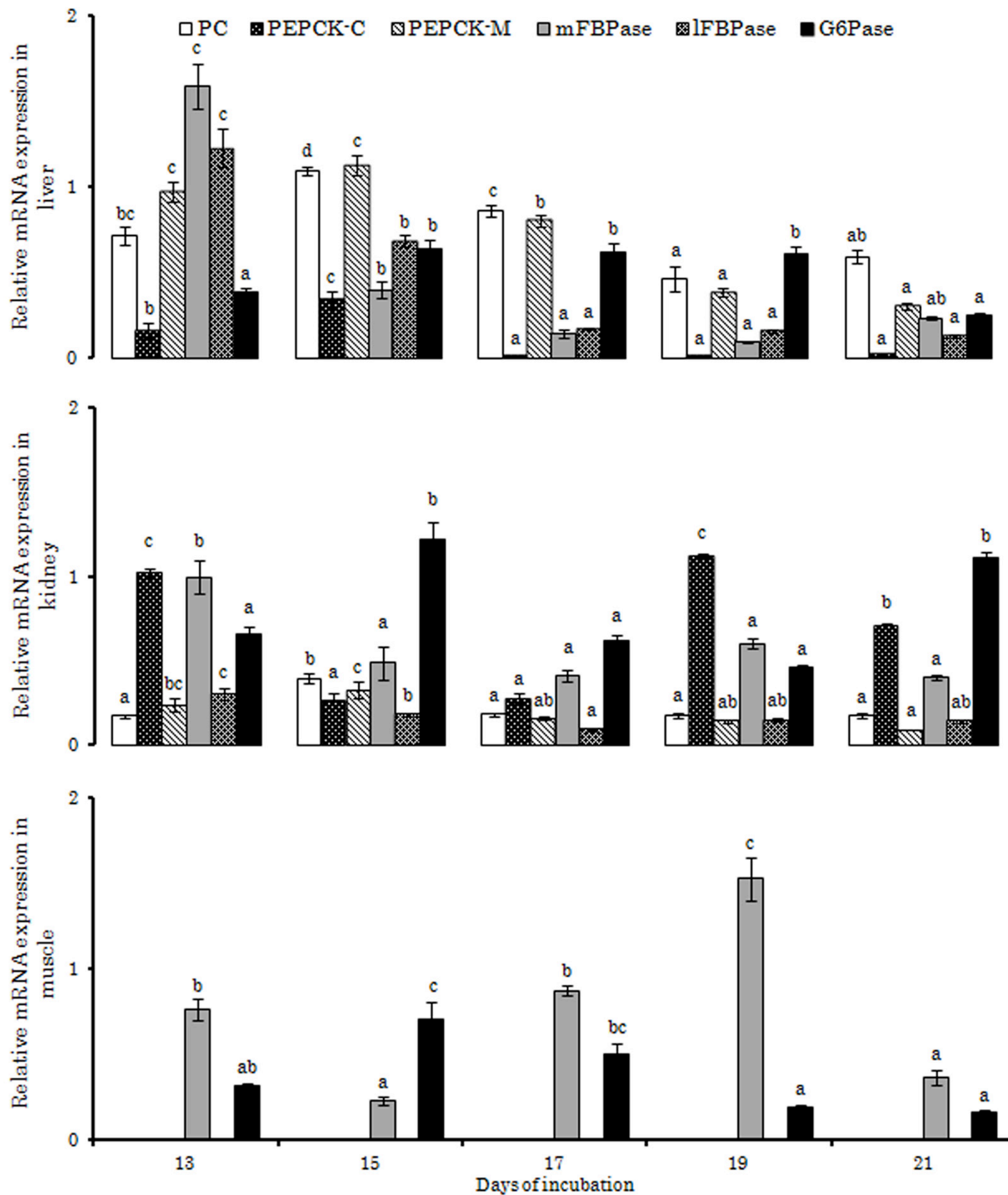


Fig 2. **Developmental changes in mRNA expression of gluconeogenic key enzymes in liver, kidney and skeletal muscle from chicken embryo.** Key enzymes: PC, pyruvate carboxylase; PEPCK-C, cytosolic isoenzyme of phosphoenolpyruvate carboxykinase, PEPCK-M, mitochondrial isoenzyme of phosphoenolpyruvate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

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 E15 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 my present data that the expressions of the enzymes had peaks between E13 and E19 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), and 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, while that in the liver remained relatively constant. 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).

My 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.

In mammals, liver isozyme of FBPase (lFBPase) is expressed predominantly in the liver and kidney, and muscle isoenzyme (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/m FBPase 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, 2002). In the liver of E18 chicken embryo, mRNA expression was detected by northern analysis (Jitrapakdee. 2002), but its changes 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, liver isozyme of FBPase (lFBPase) and G6Pase in the chicken (n=4) liver between E13 and E21. Although their experimental designs are generally

the same as mine, the embryonic ages showing highest expressions are different: E19 (Yadgary and Uni) vs. E15 (present study) in PEPCK-M and PEPCK-C, E17 vs. E13 in lFBPase and E20 vs. E15-E19 in G6Pase gene expressions. The reason for these discrepancies is not clear, but we speculate that this could be due to the difference between broiler (Yadgary and Uni) and layer (present study) chickens. It is reported that, already in embryonic ages, broiler and layer chickens are 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).

Kidney

As described in the liver section above, kidney contains both PEPCK-C and PEPCK-M (Shen and Mistry, 1979a; Shen and Mistry, 1979b; Watford *et al*., 1981) and the former isozyme enables gluconeonegesis from pyruvate, namely, amino acids that can be metabolized to pyruvate (Watford *et al*., 1981; Hod *et al*., 1986). The increased expression of PEPCK-C in the kidney on E13, E19 and E21 (Fig. 2) thus suggests a possibility that gluconeogenesis from amino acids are activated in these ages. Since protein contents of the yolk and the embryo progressively increase after E10 until before hatch (Romanoff, 1967), the increased proteins could be used for gluconeogenesis by the kidney. In addition, the increased expression of G6Pase in the kidney on E15 and E21 (Fig. 2) suggests a possible contribution of the kidney to the increase of plasma glucose level around these ages, since G6Pase metabolizes glucose-6-phosphate into glucose, thereby enables release of intracellular glucose into circulation (Stevens, 1996).

My present results of gene expressions, however, were not parallel to the previous reports of enzyme activities, where activities of PC, PEPCK-C, PEPCK-M, FBPase (Shen and Mistry, 1979a) and G6Pase (Wang, 1968; Shen and Mistry, 1979a) increased dramatically toward hatch. These activity increases seem comparable with the progressive increase of embryonic

kidney (metanephros) weight after E10 toward hatch (Junqueira, 1952). The reason for the discrepancy between gene expressions and enzyme activities remains to be clarified.

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: Annamalai *et al*., 1977) and G6Pase (enzyme activity: 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 was significantly high on E19 skeletal muscle (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 by immunohistochemistry (Hirose *et al*., 1986; Watanabe *et al*., 1986), enzyme kinetics and Western blot analyses (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). In chicken embryos, glycogen content per unit weight of the muscle gradually increases from E10 to E20 (García *et al*., 1986). We speculate from

the data of G6Pase mRNA (Fig. 2) that the embryonic skeletal muscle also releases glucose into circulation by converting accumulated glycogen into glucose or by metabolizing increased muscle lactate to glucose, thereby contributing to glucose homeostasis of chicken embryos. A confirmation of G6Pase enzyme activity of the embryonic chicken muscle is a further requisite.

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. My 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-C, and FBPase showed maximum expression either on E13 or E15 (Fig. 2), when liver glycogen content in the course of rapid increases (Ballard and Oliver, 1963; Ramanoff, 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.

Although its remain to be clear but the increased mRNA expression of some gluconeogenic key enzymes (as described in kidney section) as well as the increased expression of G6Pase on E15 and E21 (Fig. 2) suggests a possibility of ability to release intracellular glucose into circulation (Stevens, 1996).

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; Garcia *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.

To my knowledge, this is the first report where mRNA expressions of all of the four key enzymes of gluconeogenesis in the chicken embryonic liver, kidney and muscle were analyzed. The overall results suggested active gluconeogenesis in these tissues that could lead to high plasma glucose in chicken embryos.

Chapter 4

Ontogenic profile of insulin and glucagon immunoreactive cells in embryonic chicken pancreas

Introduction

Previous reports (Yarnell *et al*., 1966; Evans and Scholz, 1973; Lu *et al*., 2007) as well as my data (chapter 3) demonstrate that chicken embryos maintain high plasma glucose from the early incubation period. Insulin and glucagon are the two most important hormones for glucose metabolism secreted from pancreas. Previous studies have reported insulin cells on day 3 and glucagon cells as early as day 3 of incubation in chicken embryo (Beaupain and Dieterlen-Lievre, 1974; Dieterlen-Lievre and Beaupain, 1974). Chicken pancreas is consists of four lobes: splenic, third, dorsal and ventral (Clara, 1924; Mikami & Ono, 1962, Hodges, 1974) and the differentiation of these pancreatic lobes occurs after 10 days of incubation (Ono, 1967). Insulin and glucagon is not equally distributed in those lobes. In adult period, the concentration of glucagon is high in splenic lobe and concentration of insulin is high in ventral lobe (Foltzer *et al*., 1987). Ku *et al*. (2000) observed the distribution and relative frequencies of insulin- and glucagon-immunoreactive cells (IRC) in all those four lobes in the developing chick pancreas by using PAP methods under light microscopy and analyzed the data using an arbitrary scale. PAP and FITC methods are equally well to detect immunoglobulins in paraffin section but FITC method is slightly easier to detect very low concentration of antibody and less time consuming (Curran and Gregory, 1978). Confocal

laser scan microscope could offer greater resolution and improver rejection of out-of-focus noise than light microscopy (Kristina 2000). Moreover, analyzed data using arbitrary scale may be affected by observers' biasness (Shi *et al*, 1991, 1993). By employing of commercial software Adobe Photoshop that biasness could be eliminated (Fermin and Degraw, 1995). This study was, therefore, undertaken more precisely to understand ontogenic profile of insulin and glucagon-IRC in different lobes of pancreas by using such recently developed techniques as FITC method combined with LSM510. The data was analyzed by using Adobe Photoshop (Adobe systems, Inc., San Jose, CA, USA).

Materials and methods

Animals and sampling

The fertile eggs of white leghorn chicken from GHEN Corporation were incubated under 70% humidity and 37.8ºC. E0 was defined as the start day of incubation. Pancreas from 3-4 chicken embryos of E11 to E21 were collected after cracking under stereoscopy, fixed with Bouins solution and preserved until further analysis. All the experiments were performed in accordance with Regulation for Animal Experiments in Gifu University.

Dehydration, cleaning, and embedding:

Pancreas was first divided into 3 pieces (head, middle and tail) and then dehydrated by passing through 90%, 95% ethanol followed by 2 changes of 100% ethanol, 30 minutes each. After dehydration tissues were cleaned by passing through for 3 changes of xylene, 20 minutes each. The dehydrated tissues were infiltrated by passing through 3 different pot of pure melted paraffin at 60ºC and embedded the tissues in a paraffin block.

Sectioning, slide preparation and immunostaining:

After paraffin embedding, 8 µM serial sections were cut. Then slides were prepared with 3 representative section ribbons from 3 different parts of pancreas – one from head, one from middle part and another one from tail and 3 representative slides were selected from every sample. Slides were deparaffinized, rehydrated and cleaned by sequential treatment with xylene, ethanol and saturated lithium carbonate. Prepared slides were incubated with the insulin antiserum (a kind gift from Professor Yasutake SHIMIZU, Veterinary Physiology, Gifu University) (Tonosaki *et al.,* 2007) et al., (1:1000 dilution in PBS) or glucagon antiserum (Enzo Life Sciences, Butler Pike, USA) (1:200 dilution in PBS) for 1 hour at 37ºC. For insulin, control sections were treated with Guinea Pig serum (ROCKLAND

Immunochemicals Inc., Gilbertsville, USA) and for glucagon control sections were treated with rabbit serum. Subsequently, insulin immunostained slides were exposed to Fluorescein-Labeled Affinity Purified Antibody to Guinea Pig IgG (Kirkegaard & Perry Laboratories, Inc., Gildford, UK) and glucagon immunostained slides were exposed to Fluorescein-Labeled Affinity Purified Antibody to rabbit IgG (Kirkegaard & Perry Laboratories, Inc., Gildford, UK).

Image capture with LSM510

Fluorescence of FITC was visualized by excitation at 458, 477, 488 and 514 nm with argon laser on a confocal laser scanning microscope LSM510, ver 3.2 (Carl Zeiss Co., Ltd, New York, USA) equipped with Axiovert100M confocal laser scanning software. Images (512 × 512 or 1,024 × 1,024 pixels) were acquired through ×40 (NA 0.8) Plan-Neofluor objectives with single track mode using the same pinhole setting to obtain an optical section. All images were saved in tagged-image file format (TIFF)

Image and image analysis

Pixels of insulin or glucagon immunoreactive signals were calculated by using Adobe Photoshop CS (ver 8). In Photoshop the following procedure was maintained for all images

File > Open image > Select > Color range > Select Greens

From histogram, total pixel and pixels of IRC were taken. The following equation was used for calculation:

Percentage of any IRC = (Pixel of any IRC /Total pixel) × 100

Statistics

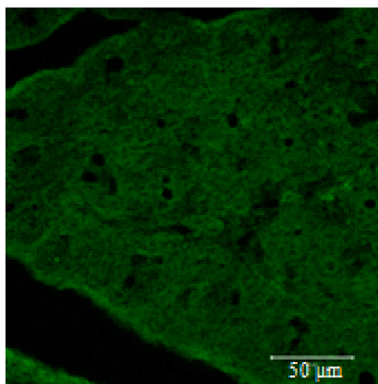
Data are presented as means ± SEM. Comparisons among groups were performed with one way ANOVA followed by Tukey's multiple comparison test using SPSS statistical package (ver. 13.00). Differences were considered statistically significant at $P < 0.05$

Results

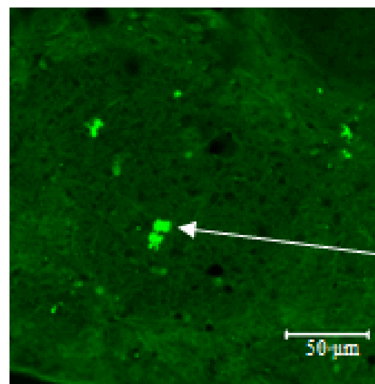
Confirmation of immunoreactivity of antibody

Insulin- and glucagon- IRC were detected in immunostained sections. No IRC were detected in control sections. Representative pictures are presented in Fig. 1

Panel A

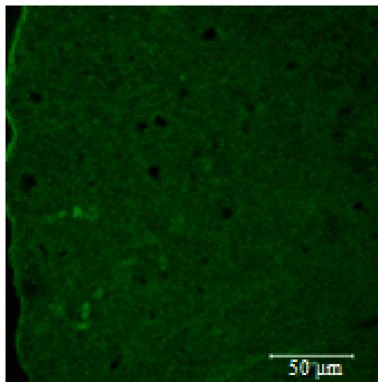


a

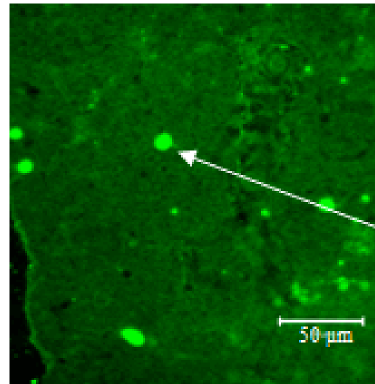


b

Panel B



a



b

Fig 1. **Panel A**. a) Control section for insulin - day 15 dorsal lobe of pancreas b) insulin immunoreactive section – day 15 dorsal lobe of pancreas. **Panel B.** a) Control section for glucagon -day 15 dorsal lobe of pancreas b) glucagon immunoreactive section – day 15 dorsal lobe of pancreas.

Immunohistochemistry data analysis

As shown in Fig. 2, all the lobes expressed both insulin- and glucagon-IRC from E11 to E21. Percentage of insulin-IRC (panel A) in every lobe of pancreas was in increasing tendency from E11 to E19, except dorsal lobe. On E17 dorsal lobe expressed lowest percentage of insulin-IRC. On E21 the expression of insulin-IRC was shown to be decreased. On the other hand, percentage of glucagon-IRC (panel B) in every lobe of pancreas was in decreasing tendency from E11 to E15. It was increased continually again from E17 to E21.

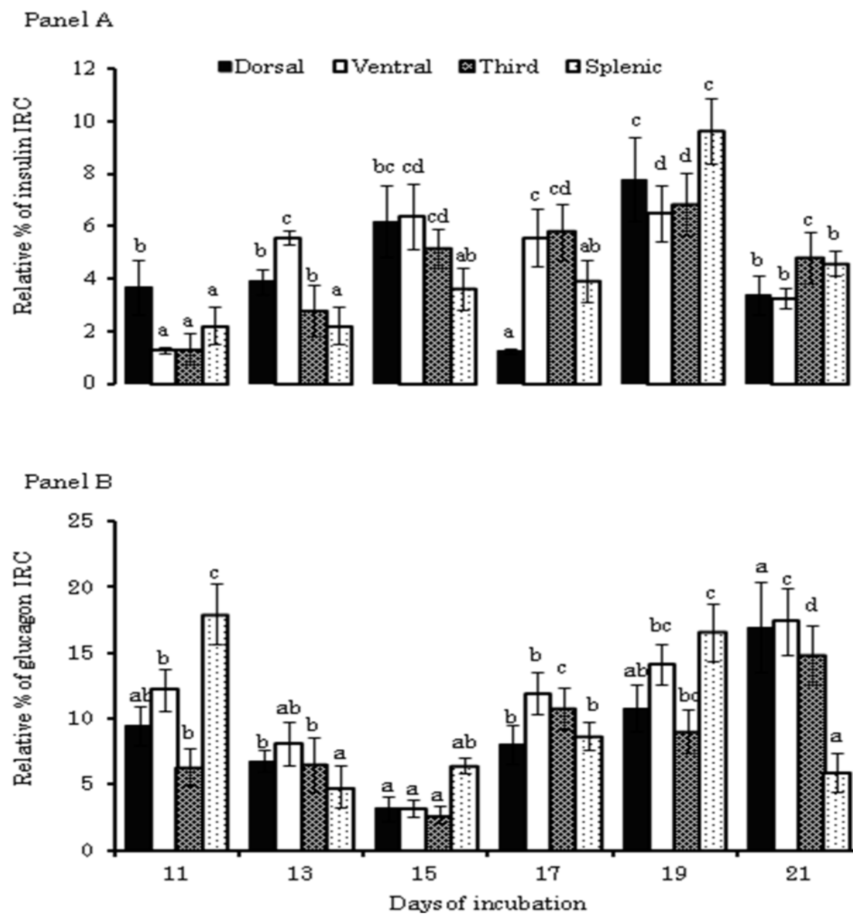


Fig 2. **Changes in percentage of insulin- IRC (panel A) and in percentage of glucagon-IRC (panel B) in dorsal lobe, ventral lobe, third lobe and splenic lobe of pancreas during chicken embryogenesis.** Pancreases (n = 5) were cut into three parts - head, middle and tail.

After paraffin embedding, sectioning, slides were prepared. Randomly selected slides were immunostained by insulin or glucagon. Then images were taken by LSM510 and analyzed by Adobe Photoshop CS (ver. 8). Different alphabets show significant difference within the same lobe (dorsal or ventral or third or splenic) by Tukey's multiple range test.

Discussion

My study demonstrated that pancreas was found into 4 differentiated lobes during the whole experimental period (E11 to E21) which was consistent with Ono's report (1967) but inconsistent with Ku *et al*. report (2000), where splenic lobe was observed from E13. Moreover, the present result depicted all the lobes that expressed both insulin- and glucagon-IRC from early period of my experiment. My present results of changes in relative % of insulin- and glucagon-IRC in different lobes also, however, were not consistent to the Ku *et al*. (2000) report. The embryonic ages showing highest expression of IRC were also different but difficult to compare as they used arbitrary scale to observe the frequencies of IRC. These differences between the pattern of changes of insulin- and glucagon- IRC of my study and Ku *et al*. could be derived in part from the difference of the chicken breed used (White leghorn in my case and not mentioned in the case of Ku *et al.*) and in part from the sensitivity of the immunohistochemical methods used (sensitive FITC combined with LSM in my case and PAP with conventional light microscopy in the case of Ku *et al.*). The difficulties of comparison was raised not only for species and technique but also for analysis

Insulin-IRC maintained an increasing tendency up to E19 (with little decrease on E17) (Fig 2), which was nearly parallel with plasma insulin level (Lu et al., 2007). But the effect of insulin on glucose homeostasis was still unclear in the chicken. A major insulin responsive glucose transporter, GLUT4, is deficient in adult chicken (Seki *et al.,* 2003) but insulin slightly stimulates the uptake of glucose in growing chicks (Tokushima *et al.,* 2005). Hohlweg et al. (1999) demonstrated that from E13, insulin appears to be much more sensitive to amino acid than to carbohydrate. De Pablo et al. (1991) addressed insulin as a growth factor in chicken embryo rather than a hormone for glucose homeostasis.

Percentage of glucagon-IRC decreased in first few days of my experimental period and reached its lowest level on E15 and then increased again until hatch (Fig 2). The tendency is

nearly parallel with plasma glucagon level (Lu *et al.,* 2007), suggesting that pancreatic glucagon content is quite parallel with its plasma concentration. The lowest level on E15 correlates with dramatic activation of glycogen synthesis around that day (Picardo and Dickson., 1982; Romanoff, 1967). It has been already well described that glucagon appears to be the dominant pancreatic hormone in birds and plays a critical role in providing the glucose requirement of chick embryos during embryogenesis (Lu *et al.,* 2007). So, high percentage of glucagon before and after E15 might induce gluconeogenesis as gluconeogenesis is active in liver of chicken embryo during these periods (Rinaudo, 1961; Ballard and Oliver, 1963; and also chapter 2).

The overall results suggested a possibility of glucagon-induced gluconeogenesis before and after E15 in liver. The effect of insulin on glucose uptake regulation should be further studied in chicken embryo.

General Discussion

I have investigated the:

1. ontogenic profile of hexokinase isoenzyme and glucokinase mRNA expression in liver and muscle
2. ontogenic profile of gluconeogenic key enzyme mRNA expression in liver, muscle and kidney
3. ontogenic profile of insulin- and glucagon-immunoreactive cells in pancreas.

The mRNA level does not always reflect the enzymatic activity as well as the immunoreactive signals of cells in tissue does not directly indicate the plasma level. The body maintains complex metabolic and endocrine function, so the day-to-day effect of enzymes and hormones on blood glucose would not be easy to explain. However, taking these difficulties into account, I will try in this chapter to discuss the overall tendency of glucose homeostasis in chick embryo. To describe the overall tendency in a comprehensible way, I have divided the experimental period in two parts: before E15 (around) and after E15 (around) as the following events happen in chicken embryo around E15:

- blood glucose level reached in high level
- glucagon level is sharply decreased
- rapid embryonic growth is started
- rapid glycogensis is started in liver
- the junction of the amnion and the chorion is ruptured

Effects of hexokinase isoenzymes, gluconeogenic key enzymes, insulin and glucagon on glucose homeostatic mechanism in liver (Figs 1 and 2)

The high percentage of glucagon-IRC in pancreas before and after E15 (chapter 4, Fig 2)

could induce gluconeogenesis in both period of time as would be evidenced by increased gluconeogenic key enzyme mRNA (chapter 3, Fig 2). Increased lactate in muscle, amniotic fluid, allantoic fluid and yolk (García *et al*., 1986; Høiby *et al*., 1987) are possible substrates of those enzymes (Figs 1 and 2). This will be followed by elevated release of glucose into circulation as G6Pase is also highly expressed around this age (chapter 3, Fig 2), which will lead high blood glucose. However, the glucose uptake regulation might be different between these two periods. Before E15, liver expresses high HKI mRNA (chapter 2, Fig 6). The HKI metabolizes glucose at its maximum speed when the glucose concentration is in its physiological range (Stevens, 1996). This suggests that, before E15, liver does not contribute to the regulation of blood glucose very much but could utilize blood glucose as its full speed to support the hepatic growth. However, after E15, liver expresses high glucokinase (GK) mRNA (chapter 2, Fig 6). GK is a standard hepatic enzyme of the adult chicken that utilizes glucose according to the glucose concentration in blood (Stevens, 1996). The induction of GK after E15 could mean that embryonic liver acquires its ability to uptake glucose according to its plasma concentration and then to convert this glucose into glycogen (glycogenesis). High percentage of insulin-IRC (chapter 4, Fig 2) correlated with high expression of hexokinase isoenzyme after E15 though the mode of action of insulin is further to be studied.

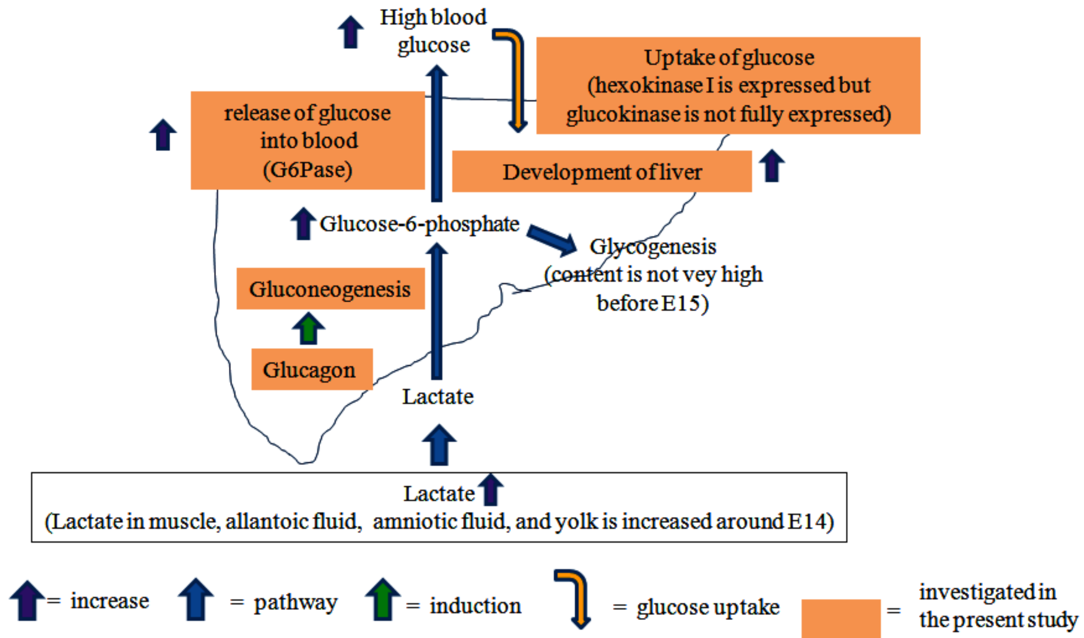


Fig 1. **Possible effects of hexokinase isoenzyme, gluconeogenic key enzyme and glucagon on glucose homeostasis before E15 (around) in liver.**

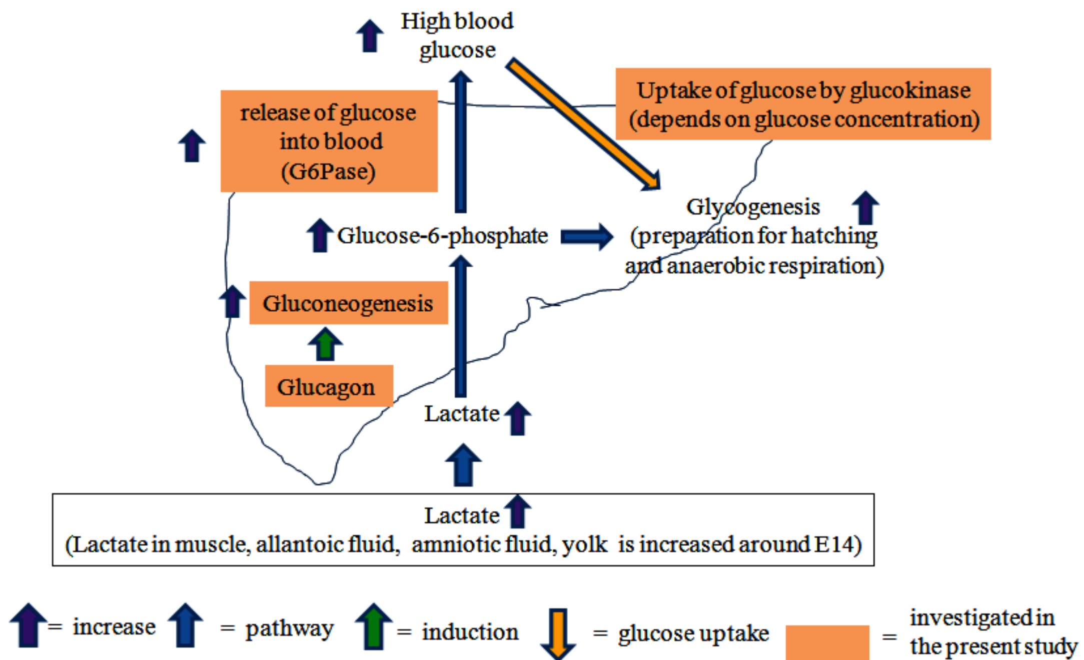


Fig 2. **Possible effects of hexokinase isoenzyme, gluconeogenic key enzyme and glucagon on glucose homeostasis after E15 (around) in liver.**

Effects of hexokinase isoenzymes, gluconeogenic key enzymes, insulin and glucagon on glucose homeostatic mechanism in muscle (Figs 3 and 4)

Although the mechanism is not determined yet, glucagon could induce gluconeogenesis from increased lactate within the muscle (García *et al*., 1986) followed by the release of some glucose into circulation with the help of G6Pase that is expressing high around E15 (chapter 3, Fig 2). As G6Pase expression decreases toward hatch (chapter 3, Fig 2), glucose release might also be decreased. Before E15, when muscle expresses high GK (chapter 2, Fig 6), uptake of glucose depends on blood glucose concentration (Fig 3). But in the later period, glucose uptake becomes less regulated by blood glucose concentration due to the increased expression of HKI and decreased expression of GK (chapter 2, Fig 6). The glucose transported into muscle might induce glycogenesis for the preparation of anaerobic metabolism around hatch (Fig 4). The role of insulin on glucose uptake should be further studied.

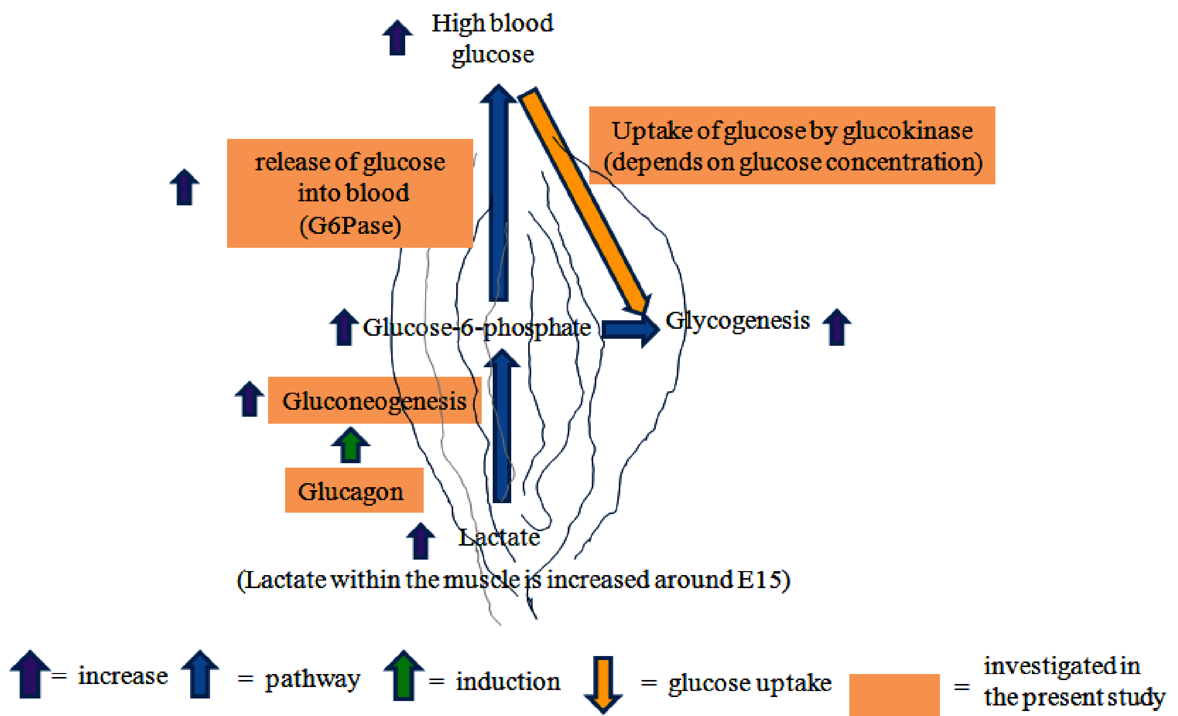


Fig 3. **Possible effects of hexokinase isoenzyme, gluconeogenic key enzyme and glucagon on glucose homeostasis before E15 (around) in muscle**

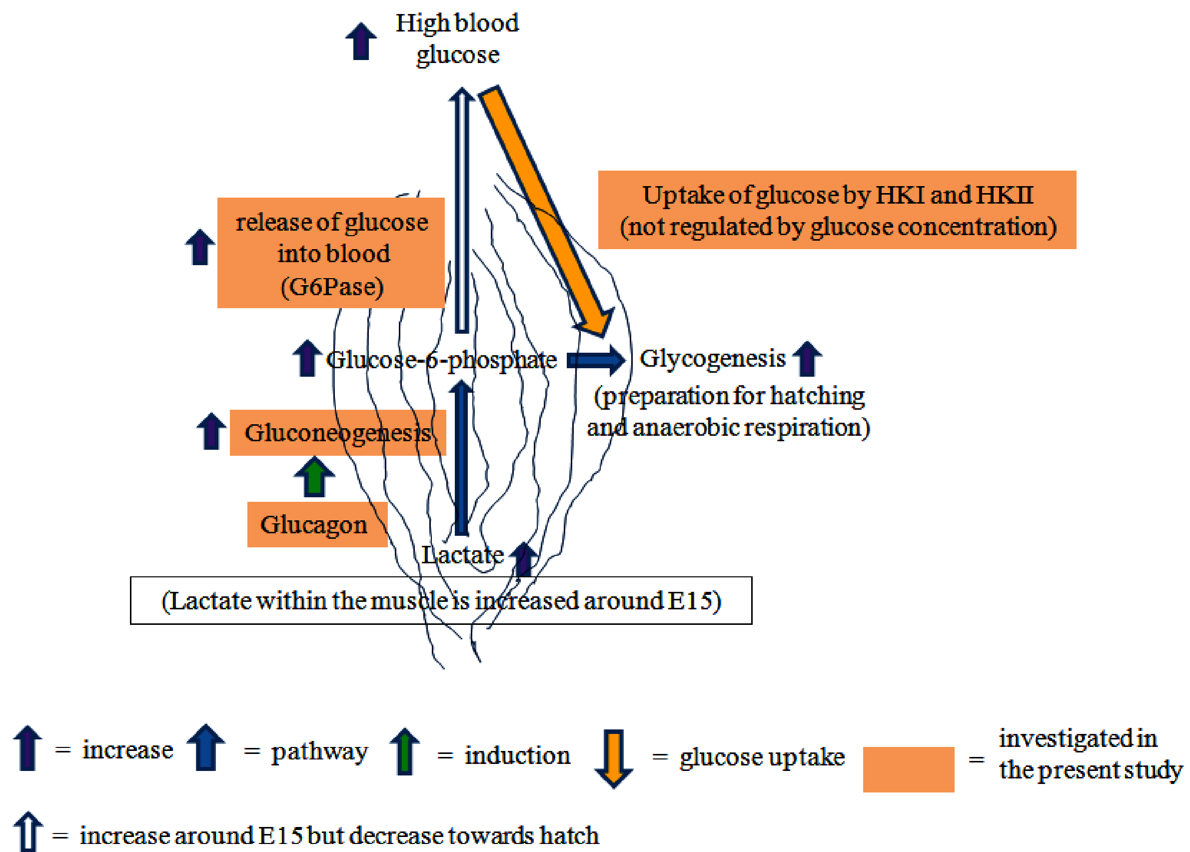


Fig 4. **Possible effects of hexokinase isoenzyme, gluconeogenic key enzyme and glucagon on glucose homeostasis after E15 (around) in muscle.**

Effects of hexokinase isoenzymes, gluconeogenic key enzymes, insulin and glucagon on glucose homeostatic mechanism in kidney (Fig 5)

I discussed the effect of gluconeogenic key enzymes, insulin and glucagon on both periods (after and before E15) in one figure (Fig 5) because tendency is almost the same on those periods. It has been reported that G6Pase activity in kidney is detected from E8 followed by rapid increase during development (Wang, 1968). My mRNA level data (chapter 3, Fig 2) was not parallel to the data of Wang (1968), which is remained to be clear. However, increased expression of PEPCK-C mRNA on E13, E19 and E21 and G6Pase mRNA on E15 and E21 (chapter 3, Fig 2) suggest a possibility of increased gluconeogenesis and glucose release into circulation on both periods of time. Here, the substrate could be increased amino

acids in egg yolk from E10 (Romanoff, 1967). The tendency also correlates with increased glucagon before and after E15. It should also be noted that at E13, the junction of the amnion and the chorion raptures and the albumen flow into the amniotic fluid. As the embryo swallows amniotic fluid, it absorbs more than 70% of the protein reserves of the albumen through the gut. Hohlweg *et al.,* (1999) demonstrated that insulin appear to be much more sensitive to amino acid levels than to carbohydrate levels and insulin is the key hormone regulating the concentration of amino acids and related compounds in plasma, amniotic fluid, and allantoic fluid of E13 chicken embryos. As body regulates amino acid metabolism and glucose metabolism simultaneously, insulin may have some secondary effect on glucose homeostasis (Fig 5).

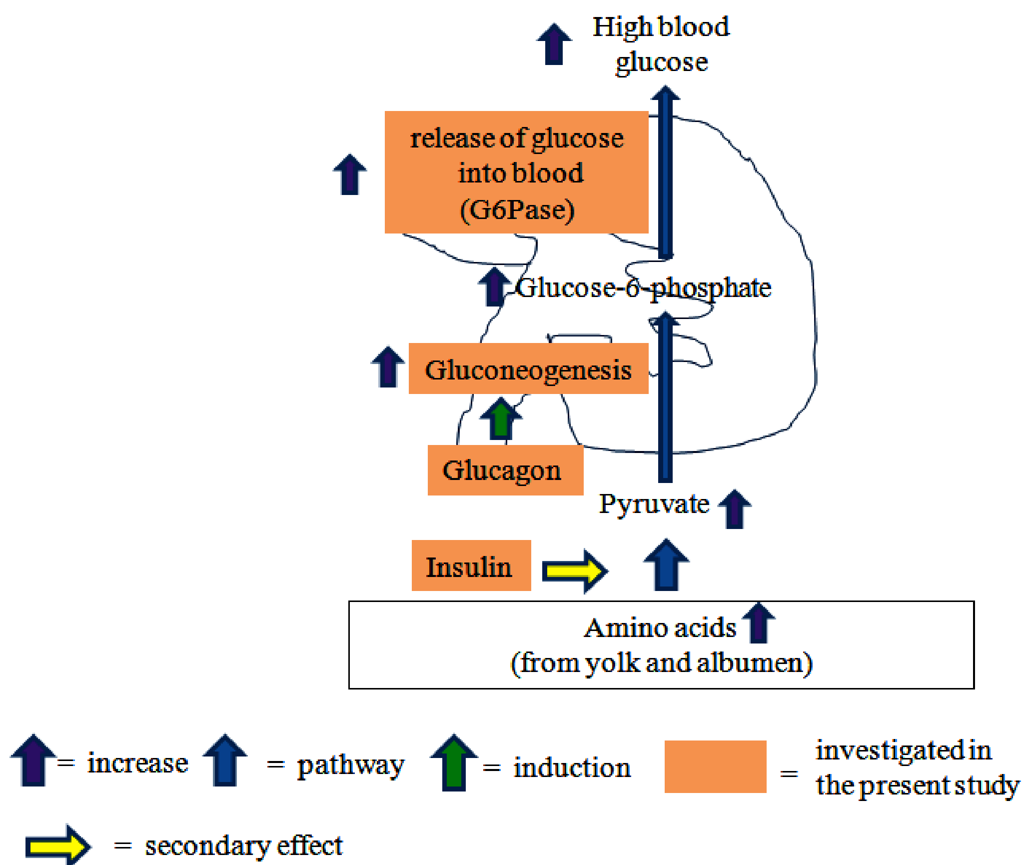


Fig 5. **Possible effects of hexokinase isoenzyme, gluconeogenic key enzyme, insulin and glucagon on glucose homeostasis before and after E15 (around) in kidney.**

To conclude this chapter, although day-to-day effects of investigated enzymes and hormones on blood glucose level could not be thoroughly explained in the present study, it was suggested that before and after E15, glucagon induced gluconeogenic enzymes in liver, kidney and muscle to some extent and thereby induced gluconeogenesis. Glucagon could also induce glucose release into circulation in these periods. Although the role of insulin on glucose uptake needs further study, it might have some secondary role as insulin induces the metabolism of amino acids. All the questions not resolved in the present study are worth clarifying and it will contribute to reveal the mechanisms of increasing blood glucose during incubation, leading to the clarification of the mechanisms underlying high blood glucose of the laying hens.

General Summary

Chicken maintains a blood glucose level that is twice as high as that in most mammals. Not only adult chicken but also chicken embryo maintains high level from the second week of incubation although their main source of fuel is lipid and protein. Liver is the major organ for maintaining plasma glucose. Kidney, muscle, brain, pancreas play some roles as well. In another point of fact, many factors affect the regulation of blood glucose levels: glucose transporters, hexokinase isoenzymes, gluconeogenic enzymes, glycogenic and glycogenolytic enzymes, pancreatic hormones etc.

So, I have investigated:

- ontogenic profile of hexokinase isoenzyme gene expressions in liver and muscle
- ontogenic profile of gluconeogenic key enzyme gene expressions in liver and muscle and kidney
- ontogenic profile of insulin- and glucagon immunoreactive cells in pancreas and tried to clarify the effect of them on glucose homeostasis.

Glucose is transported into the cell from the blood by glucose transporters (GLUT) and thereafter catalyzed into glucose-6-phosphate by hexokinases which makes glucose available for further utilization. This catalytic reaction has a large negative free energy change (ΔG = 16.7 kJ/mol) and is an important control point of glycolysis and glycogenesis. In mammals, HK-I mRNA is widely distributed in all vertebrate tissues and has high affinity for glucose and HK-II is predominantly expressed in insulin sensitive tissues like muscle and adipose tissues. GK has the most distinctive properties with half molecular mass (50 kDa) of other HKs (100 kDa) and low affinity for hexoses (Km 0.5~10 mM). HKI and HKII in most cells follow the Michaelis-Menten kinetics and in contrast GK displays sigmoidal kinetics.

To clarify the role of hexokinase isoenzymes in White Leghorn chicken embryo - the ontogenic profile of mRNA expressions of hexokinase isoenzymes was observed in liver and muscle (n = 6) by semi-quantitative reverse transcription PCR. In the liver, HKI mRNAs were gradually decreased during the experimental period, while GK mRNAs were gradually increased. In the skeletal muscle, HKI increased on day 13 and was almost stable thereafter, while GK was gradually decreased. HKII expression tended to increase on day 13 and remained stable thereafter in both the organs. These results suggest some possibilities 1) expression profiles of HK isoenzymes differ between embryonic and non-embryonic chickens, 2) well-established domination of GK to HK in the liver is gradually acquired during embryogenesis and liver acquires its role in blood glucose homeostasis 3) in some stages of development skeletal muscle plays a liver-like role in glucose homeostasis. Whether embryonic liver expresses active HKI protein and embryonic skeletal muscle expresses active GK protein should be studied further

As chicken maintains high plasma glucose from the second week of incubation, it is expected that gluconeogenic pathway may active from early of embryo development. To elucidate biochemical mechanisms underlining high blood glucose in chicken embryo, mRNA expression profiles of gluconeogenic key enzymes (pyruvate carboxylase (PC), cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C), mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M), muscle fructose-1,6-bisphosphatase (mFBPase), liver fructose-1,6-bisphosphatase (lFBPase), and glucose-6-phosphatase (G6Pase) in liver, kidney and skeletal muscle were characterized (n = 8) by real-time PCR. All the enzymes were expressed in the liver and kidney, 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 high between E15 and E19. In the kidney, expressions of the enzymes were high either on E13 or E15. In addition PEPCK-C showed high expression also on E19 and E21 and G6Pase showed high expression also on E21. In the skeletal muscle, mFBPase

peaked on E19 and G6Pase peaked on E15. These results suggest that gluconeogenesis is active in chicken embryos from the early period of development but regulations of mRNA expressions of the gluconeogenic key enzymes are different in liver, kidney and skeletal muscle. Liver may utilize increased lactate, whereas kidney may utilize increased protein of egg as a substrate for gluconeogenesis and enables increased release of glucose into circulation. The results also suggest that 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.

Insulin and glucagon are two most important hormones for glucose metabolism which are secreted from pancreas. Pancreas consists of four lobes: splenic, third, dorsal and ventral. Differentiation of these pancreatic lobes occurs after 10 days of incubation. To clarify ontogenic profile of insulin and glucagon immunoreactive cells (IRC) in different lobes of pancreas (n = 5) percentage of IRC were characterized by using FITC method and LSM510. The data was analyzed by using Adobe Photoshop. All the lobes expressed both insulin- and glucagon-IRC from the early period of my experiment. Insulin-IRC maintained an increasing tendency up to E19 which was nearly parallel with plasma insulin level (Lu et al., 2007). Although the effect of insulin on glucose homeostasis was still not clear in the chicken but some reports (De Pablo et al., 1991) addressed that insulin may play a growth factor role in chicken embryo. Glucagon-IRC decreased its lowest level on E15 and then increased again and continued until hatch (Fig 2). High percentage of glucagon before and after E15 might induce gluconeogenesis and glycogenesis (Picardo and Dickson., 1982; Romanoff, 1967; Rinaudo, 1961; Ballard and Oliver, 1963).

Finally, my results suggested that 1) liver play the key role for glucose homeostasis in chicken embryo but in some stages of development skeletal muscle may play a liver-like role. 2) kidney could contribute to increase plasma glucose at some stages of development 3)

insulin and glucagon express from the early embryonic development in all four lobes of pancreas 4) although the role insulin should be clarified but glucagon may play the key role for maintaining glucose homeostasis.

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Academic papers relating the dissertation

Tushar K. Roy, Atsushi Iwasawa, Yasutake Shimizu, Koji Kageyama and Norio Yoshizaki. Ontogenic Profile of Glucokinase and Hexokinase mRNA Expressions in Embryonic Chicken Liver and Muscle. The Journal of Poultry Science, Volume 50, Issue 3, Pages 270-274, 2013.

Tushar K. Roy, Atsushi Iwasawa, Yasutake Shimizu, Koji Kageyama and Norio Yoshizaki. Ontogenic Profile of Gluconeogenic Key Enzyme Gene Expressions in Embryonic Chicken Liver, Kidney and Muscle. The Journal of Poultry Science, Volume 50, Issue 4, pages 380-387, 2013.

Acknowledgements

I would like to express my deepest gratitude, profound respect to my supervisor, YOSHIZAKI Norio, Professor of Faculty of Applied Biological Science, Gifu University, for kindly accepting me as a PhD student and excellent co-operation in conducting my research. His valuable advice and co-operation at every stage made the work easier and interesting to me.

I wish to express my gratitude and whole hearted thanks to Professor IWASAWA Atsushi and letting me be a part of his comparative biochemistry research group. I am grateful for his continuous encouragement, time and all-out effort for my academic development. I most gratefully acknowledge that his untiring and generous help and encouragement to accomplish this study. Really, not only as an academic advisor but also a complete philosopher he guided and helped me both academic and non-academic ways which I must have to acknowledge in this opportunity.

I am very much grateful and express my sincerest and deepest sense of indebtedness to my co-supervisor Professor KOHSAKA Tetsuya for his kind and valuable advises which make my sense of research broader.

I want to convey my deepest thanks to Professor SASANAMI Tomohiro and Associate Professor YAYOTA Masato for their kind help and suggestions. Their kind and valuable suggestions really helped me to update my thesis paper.

I want to express my deepest thanks and sincere gratitude to Professor KAGEYAMA Koji for his sincere and kind help in conducting some of my experiments in his laboratory. His valuable suggestions during my experiment helped me a lot undoubtedly.

I wish to express my sincere thanks to TAKAHASHI Saki, KUNO Kyoko, TAKEZAKI Yuya, FUJII Haruka, Nojebuzzaman, Shahin Al Mamun, SHIBATA Mitsuhiro for their help

in the development of basic foundation of this study and for close assistance in carrying out experiments and data analysis. I have had the pleasure of working with them.

I am also very thankful to the staff of Genomic Research Division and Gifu university Field Science Center for cordial and continuous support during experiment.

I extend heartfelt thank to my friend Afroza Sultana and her family who’s hearties company, cordial cooperation and all kinds of supports leads me to come Japan and carry out this study smoothly and successfully.

I express my deepest thanks to my wife Joyti Barma for her unbelievable sacrifices, to allow me in devoting this study and for continuous support during my research work.

Finally, I owe special thanks to my all friends specially Tanveer Tazib, Kazal Boron Biswas, Asif Mahmud, Jahir Raihan, MAJ Lincoln and their family for their help and co-operation and made my life enjoyable in Japan.

Appendix

Composition of the reagents

Phosphate buffer saline (PBS)

80 gm NaCl, 20 gm KCl, 14.4 gm Na_2HPO_4 and 2.4 gm KH_2PO4 were dissolved in 800 ml of distilled water and toping up to 1 liter. And the p^H was adjusted to 7.5. The stoke solution was diluted accordingly before use of each experiment.

EDTA solution

EDTA2Na.2H_2O 372.24 gm was taken into a beaker and made it up to 400 ml. Then the p^H was adjusted to 8.00 by adding NaOH (about 10 gm). After adjusting the p^H at 8.00 the sample volume was made upto 500 ml with distilled water. Finally it was autoclaved for 20 minutes at 120ºC.

70% ethanol

700 ml absolute ethanol (Japanese alcoholic sales Ltd.) was mixed with distilled water and was made it up to 1000 ml. It was preserved in the room temperature.

Membrane binding solution

1 liter of membrane binding solution was prepared with 4.5M guanidine isothiocyanate and 0.5M potassium acetate.

95% ethanol

95 ml Ethanol (Nacalai tesque, Inc, Japan.) was mixed with autoclaved distilled water and make it up to 100 ml and was preserved it below 4ºC until use.

Membrane wash solution

Just before experiment membrane binding solution from Promega Corporation was mixed with 95% ethanol.

5 M EDTA

EDTA2Na.2H_2O 93.06 gm was taken into a beaker and made it up to 400 ml. Then the p^H was adjusted up to 8.00 by adding NaOH about 10 gm. After adjusting the p^H at 8.00 it was made upto 500 ml with distilled water and it was also autoclaved.

10×TBE buffer

Tris 60.55 gm and borate 30 gm was taken into a beaker and 0.5 M EDTA (p^H 8.00) 40 ml was poured in the beaker and made it up to 1 liter by adding distilled water. After mixing it was autoclaved.