JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.0170004

≪Research Note≫

Fasting and Glucagon Stimulate Gene Expression of Pyruvate Dehydrogenase Kinase 4 in Chickens

Kazuhisa Honda*, Shoko Takagi*, Kiyotaka Kurachi, Haruka Sugimoto, Takaoki Saneyasu and Hiroshi Kamisoyama

Graduate School of Agricultural Science, Kobe University, Kobe 657−8501, Japan

The excessive accumulation of body fat has become a serious problem in the broiler industry. However, the molecular mechanisms underlying the regulation of lipid metabolism-related genes in broiler chickens are not fully understood. In the present study, we investigated the role of glucagon on the expression of lipid metabolism-related genes in chicken white adipose tissue (WAT). Four hours of fasting significantly increased plasma levels of free fatty acid in broiler chickens. The mRNA levels of adipose triglyceride lipase (ATGL) and pyruvate dehydrogenase kinase 4 (PDK4) in abdominal WAT significantly increased by fasting, whereas the mRNA levels of diacylglycerol O-acyltransferase homolog 2 (DGAT2) and peroxisome proliferator-activated receptor-γ (PPARγ) significantly decreased. The results suggest that fasting stimulates lipolysis and suppresses adipogenesis and re-esterification of TG in chicken WAT. Glucagon significantly increased the mRNA levels of PDK4 in chicken primary adipocytes, whereas there were no significant changes in the mRNA levels of ATGL, DGAT2, and PPARγ. Our findings suggest that glucagon upregulates PDK4 expression and may stimulate lipolysis without affecting the expression of ATGL in chicken WAT.

Key words: ATGL, chicken, free fatty acid, glucagon, PDK4

J. Poult. Sci., 54: 292−295, 2017

Introduction

The accumulation of body fat causes various metabolic disorders in modern broiler chickens (Julian *et al*., 2005; Richards and Proszkowiec-Weglarz, 2007). However, species-specific differences in the accumulation of body fat are reported between mammals and birds. For example, a number of hormones are known to participate in the regulation of lipolysis, but insulin is quantitatively and qualitatively the most relevant in mammals (Frühbeck *et al*., 2014). On the other hand, physiological roles of insulin in chicken white adipose tissue (WAT) appear weak or questionable (Tokushima *et al*., 2005; Scanes, 2009; Dupont *et al*., 2012). Catecholamines also stimulate lipolysis in mammals, but in birds, glucagon is the most potent stimulator of lipolysis (Langslow and Hales, 1969; Freeman and Manning, 1974; Scanes, 2009). However, the molecular mechanisms underlying the regulation of lipid metabolism-related genes in chicken WAT have not been fully elucidated.

Recent findings suggest that insulin and glucocorticoid regulate the expression of lipid metabolism-related genes in chickens. For example, diacylglycerol O-acyltransferase homolog 2 (DGAT2), which catalyzes the final step in triglyceride (TG) esterification, was downregulated by insulin neutralization in chickens, whereas pyruvate dehydrogenase kinase 4 (PDK4) was significantly upregulated (Ji *et al*., 2012). PDK4 null mice show a lower capacity for *de novo* fatty acid synthesis (Hwang *et al*., 2009). It is therefore likely that insulin stimulates TG accumulation in chicken WAT.

Adipose triglyceride lipase (ATGL), the rate limiting enzyme of TG hydrolysis in mammals, is expressed in chicken WAT, and its mRNA and protein levels are increased by artificial glucocorticoid dexamethasone *in vivo* and *ex vivo* (Serr *et al*., 2011). Dexamethasone injection also elevated plasma free fatty acid (FFA) levels (Serr *et al*., 2011). It seems likely that glucocorticoids stimulate TG hydrolysis in chicken WAT. However, the effect of glucagon on the expression of lipid metabolism-related genes, such as DGAT2, PDK4, and ATGL, in WAT has not been examined, although it is known that glucagon is the major lipolytic hormone in chickens (Goodridge, 1968; Oscar, 1991, Scanes, 2009). There is evidence that fasting significantly elevated plasma glucagon levels in broiler chickens (Dupont *et al*., 2008; Richards and McMurtry, 2008; Christensen *et al*., 2013). We previously reported that fasting significantly increased

Received: January 5, 2017, Accepted: February 21, 2017
Released Online Advance Publication: March 25, 2017
Correspondence: Kazuhisa Honda, Ph. D., Department of Bioresource Science, Graduate School of Agricultural Science, Kobe University Kobe 657−8501 Japan. (E-mail: honda@tiger.kobe-u.ac.jp)
* These authors contributed equally to this study

the mRNA levels of ATGL in WAT in broiler chicks (Saneyasu *et al*., 2013). These findings raised the hypothesis that glucagon regulates the gene expression of DGAT2, PDK4, and ATGL in chicken WAT.

In the present study, we examined the effect of fasting or glucagon on the mRNA levels of DGAT2, PDK4, and ATGL *in vivo* or *ex vivo* using broiler chickens. We also analyzed the mRNA levels of PPARγ, because PDK4 is one of the target genes of PPARγ (Sears *et al*., 2007). Our findings suggest that glucagon stimulates PDK4 expression and may stimulate lipolysis without affecting ATGL expression in chicken WAT.

Materials and Methods

Animals

Day-old male broiler chicks (ROSS 308) were purchased from a local hatchery (Ishii Co., Ltd. Tokushima, Japan). They were given free access to water and a commercial chick starter diet (Nippon Formula Feed Mfg. Co., Ltd., Kanagawa, Japan). This study was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulation.

Experiment 1. Effect of Fasting on the Expression of Lipid Metabolism-related Genes in Chickens

Eighteen 10-day-old male broiler chicks were weighed, allocated to three groups and fasted for 0 (control), 2 or 4 h prior to euthanasia by decapitation. Blood was collected from carotid artery. Plasma was separated immediately by centrifugation at 3,000×*g* for 10 min at 4℃, and plasma concentrations of FFA and glucose were measured using commercial kits (LabAssay^{TM} NEFA and LabAssay^{TM} glucose, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The abdominal WAT was excised, weighed, and frozen immediately using liquid nitrogen for real-time PCR analysis.

Experiment 2. Effect of Glucagon on the Expression of TG Metabolism-related Genes in Primary Chickens White Adipocytes

10-day-old chicks were euthanized by decapitation, and the abdominal WAT was excised. Adipocytes were isolated as described previously (Oscar *et al*., 1992), and then incubated with the incubation medium Dulbecco's Modified Eagle Medium (DMEM, 1.0 g/*l* glucose with L-glutamine and sodium pyruvate, 08456−65, Nacalai tesque, Inc.) containing 25 mM HEPES, 80 μg/m*l* kanamycin, and 3% bovine serum albumin, supplemented with either 0 (control) or 4 nM chicken glucagon for 2 h. After removing the cell culture medium, cells were washed twice with PBS, and used for real-time PCR analysis. FFA concentration in the cell culture medium was measured using a commercial kit as described in Experiment 1.

Real-time PCR Analysis

Total RNA was extracted from the WAT and adipocytes using Sepazol-RNA I (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from 2 μg of DNase I (Ambion Inc., Austin, Texas, USA)-treated total RNA using a ReverTra Ace® qPCR RT Kit (TOYOBO CO. LTD., Osaka, Japan). Complementary DNAs of chicken ATGL and PPARγ were amplified with the primers as described previously (Saneyasu *et al*. 2013). Complementary DNAs of chicken DGAT2 (GenBank accession no. XM_419374), and PDK4 (GenBank accession no. NM_001199909) were amplified with the primers as follows: DGAT2 sense, 5′-TGA ACC GTG ACA GCA TAG ACT ACA-3′; DGAT2 antisense, 5′-CCA CGA TGA TGA TGG CAT TG-3′; PDK4 sense, 5′-AGT CTG CTT CCA AAC ATT ACC AAA C-3′; PDK4 antisense, 5′-CAG TCT GCT TTG GAC CTT TAC TTG-3′. As an internal standard, complementary DNA of chicken ribosomal protein S17 (RPS17) was also amplified with the primers as described previously (Honda *et al*., 2015). THUNDERBIRD^{TM} SYBR® qPCR Mix was purchased from TOYOBO CO. LTD. (Osaka, Japan), and mRNA expression was quantified in duplicate using the Applied Biosystems 7300 Real-Time PCR system according to the supplier's recommendations.

Data Analysis

Data from Experiment 1 were analyzed by Dunnet's test. Significant differences were defined as $P<0.05$ when compared with 0 h of fasting. Data from Experiment 2 were analyzed by Student's *t*-test. All statistics was performed using a commercial software package (StatView version 5, SAS Institute, Cary, North Carolina, USA, 1998).

Results and Discussion

The mRNA levels of PDK4 and ATGL significantly increased after 4 h of fasting in WAT (Fig. 1). In contrast, the mRNA level of DGAT2 and PPARγ significantly decreased after of 2 h of fasting (Fig. 1). The plasma FFA levels significantly elevated after 4 h of fasting (Fig. 2). The plasma glucose levels significantly decreased after 4 h of fasting (Fig. 2). These findings suggest that 4 h of fasting induces lipolysis and inhibits lipogenesis in WAT in chickens.

Since glucagon is the major lipolytic hormone in chickens (Scanes, 2009), we next examined the effects of glucagon on lipid metabolism-related gene expression in adipocytes *ex vivo*. The mRNA level of PDK4 in primary white adipocytes was significantly increased by glucagon (Fig. 3). There is evidence that fasting significantly elevated plasma glucagon levels in broiler chickens (Dupont *et al*., 2008; Richards and McMurtry, 2008; Christensen *et al*., 2013). Therefore, it is possible that glucagon is involved in the increase of the PDK4 mRNA level by fasting in chicken WAT.

The mRNA levels of DGAT, ATGL, and PPARγ were not changed by glucagon (Fig 3), although FFA levels were significantly elevated by glucagon (control, 7.5±0.5 μEq/100 m*l*; glucagon, 80.1±2.2 μEq/100 m*l*; $P<0.05$), which suggests that glucagon may induce lipolysis with the elevation of the enzymatic activity of ATGL in chicken WAT.

In this study, glucagon did not affect the mRNA level of ATGL in white adipocytes (Fig. 3), although FFA levels were significantly elevated by glucagon. In mammals, ATGL activity is regulated through interactions with activator protein comparative gene identification-58 (CGI-58) and inhibitor protein G(0)/G(1) switch gene 2 (G0S2) (Frühbeck *et al*., 2014). The activation of ATGL by the interaction of

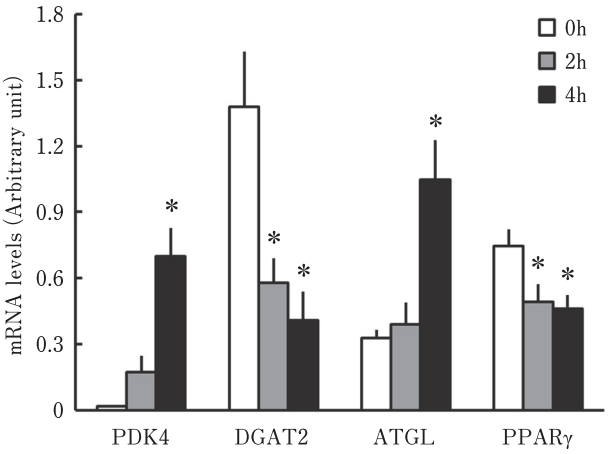

Fig. 1. **Effects of fasting on the mRNA levels of lipid metabolism-related genes in chicken white adipose tissue.** Data are means±S.E.M. (n=6). *, Significant with respect to 0 h in the fasting group (P<0.05).

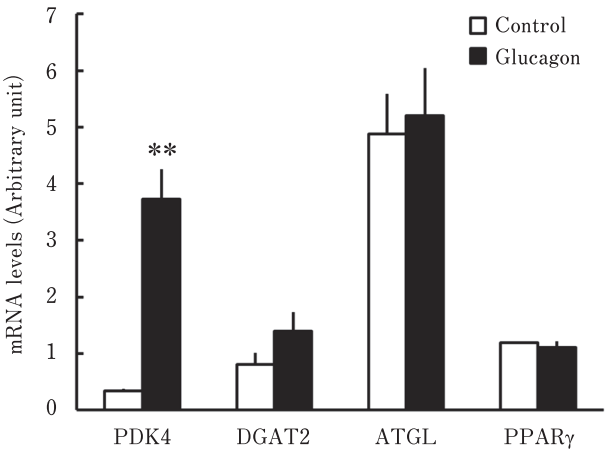

Fig. 3. **Effects of glucagon on the mRNA levels of lipid metabolism-related genes in chicken primary white adipocytes.** Data are means±S.E.M. (n=4). **, Significant with respect to the control group (P<0.01).

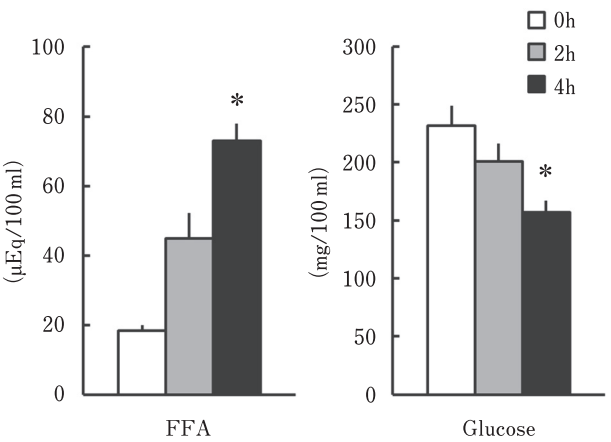

Fig. 2. **Effects of fasting on plasma levels of free fatty acid and glucose in chicks.** Data are means±S.E.M. (n=6). *, Significant with respect to 0 h in the fasting group (P<0.05).

CGI-58 and G0S2 can be induced by the elevation of intracellular cAMP levels (Frühbeck *et al*., 2014). Chicken adipocytes responded to glucagon with an increase in lipolysis and a sustained rise in cAMP (Kitabgi *et al*., 1976; Malgieri *et al*., 1975). It is therefore likely that ATGL in chicken WAT is activated by glucagon without affecting the transcription.

In this study, we provide new evidence showing that glucagon upregulates the mRNA levels of PDK4 in chicken WAT. There is no evidence demonstrating that glucagon upregulates PDK4 expression in mammals and birds. Ji *et al*. (2012) reported that PDK4 was significantly upregulated by insulin neutralization or fasting. Fasting significantly decreased plasma insulin and elevated plasma glucagon in broiler chickens (Dupont *et al*., 2008; Richards and McMurtry, 2008; Christensen *et al*., 2013). It is therefore likely that the transcription of PDK4 is negatively and positively regulated by insulin and glucagon, respectively.

In mammals, the expression of the PDK4 gene was strongly induced by PPARγ agonists (Sears *et al*., 2007). In the present study, fasting decreased the mRNA level of PPARγ (Fig. 1). However, FFA can bind and activate PPARs (Nakamura *et al*., 2014). It is therefore possible that glucagon stimulates lipolysis in chicken WAT and increases intracellular FFA, which in turn results in the activation of PPARγ and stimulation of the PDK4 gene transcription. *In vitro* experiments using PPARγ agonists will provide the direct evidence for the involvement of PPARγ and the expression of PDK4 gene in chickens.

As shown in Fig. 1, fasting altered mRNA levels of ATGL and DGAT2. However, glucagon did not alter these mRNA levels in white adipocytes (Fig. 3). The injection of insulin antibody downregulated DGAT2 expression in chicken WAT (Ji *et al*., 2012). Serr *et al*. (2011) reported that ATGL expression is stimulated by glucocorticoid in chicken WAT. Fasting decreases plasma insulin levels and increases plasma glucagon and glucocorticoid levels in chickens (Harvey and Klandorf, 1983; Dupont *et al*., 2008; Richards and McMurtry, 2008; Christensen *et al*., 2013). These findings and our results suggest that glucagon, insulin, and glucocorticoid coordinately regulate the transcription of lipid metabolism-related genes in chicken WAT.

Glucose was the predominant source of glycerol-3-phosphate in WAT in mice fed a high carbohydrate diet under the feeding condition (Chen *et al*., 2005). On the other hand, under the fasting condition, glyceroneogenesis, in contrast to glucose, is quantitatively the predominant source of glycerol-3-phosphate in the WAT of rats (Nye *et al*., 2008). In fact, fasting caused a significant increase in lipolysis but did not influence the absolute amount of FFA re-esterification (Wang *et al*., 2003). Recent findings suggest that upregulation of

PDK4 expression plays an important role in supplying glycerol-3-phosphate for the re-esterification into TG of FFA arising from lipolysis in WAT during fasting in rats (Cadoudal *et al*., 2008). There is evidence that PDK4 activity is regulated not only by gene expression but also by allosteric effectors (Jeong *et al*., 2012). In the present study, we did not measure the enzymatic activity of PDK4. Therefore, the enzymatic activity of PDK4 may not coincide with the mRNA levels. However, increased mRNA levels of PDK4 by fasting may be responsible for the glycerol-3-phosphate supply to re-esterify fatty acids liberated by glucagon-induced lipolysis in chicken WAT.

In summary, we examined the effect of fasting or glucagon on lipid metabolism-related gene expression in chicken WAT or adipocytes. Our findings suggest that glucagon upregulates PDK4 expression and may stimulate lipolysis without affecting the expression of ATGL in WAT in broiler chickens. This study provides new insight into the role of glucagon in the metabolic process in chicken WAT.

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