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Effect of 24 h Fasting on Gene Expression of AMPK, Appetite Regulation Peptides and Lipometabolism Related Factors in the Hypothalamus of Broiler Chicks

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ABSTRACT: The 5'-adenosine monophosphate-activated protein kinase (AMPK) is a key part of a kinase-signaling cascade that acts to maintain energy homeostasis. The objective of this experiment was to investigate the possible effects of fasting and refeeding on the gene expression of hypothalamic AMPK, some appetitive regulating peptides and lipid metabolism related enzymes. Seven-day-old male broiler (Arbor Acres) chicks were allocated into three equal treatments: fed *ad libitum* (control); fasted for 24 h; fasted for 24 h and then refed for 24 h. Compared with the control, the hypothalamic gene expression of AMPKα2, AMPKβ1, AMPKβ2, AMPKγ1, Ste20-related adaptor protein β (STRADβ), mouse protein 25α (MO25α) and agouti-related peptide (AgRP) were increased after fasting for 24 h. No significant difference among treatments was observed in mRNA levels of AMPKα1, AMPKγ2, LKB1 and neuropeptide Y (NPY). However, the expression of MO25β, pro-opiomelanocortin (POMC), corticotropin-releasing hormone (CRH), ghrelin, fatty acid synthase (FAS), acetyl-CoA carboxylase α (ACCα), carnitine palmitoyltransferase 1 (CPT-1) and sterol regulatory element binding protein-1 (SREBP-1) were significantly decreased. The present results indicated that 24 h fasting altered gene expression of AMPK subunits, appetite regulation peptides and lipometabolism related factors in chick's hypothalamus; the hypothalamic FAS signaling pathway might be involved in the AMPK regulated energy homeostasis and/or appetite regulation in poultry. (**Key Words:** Metabolism, Nutrition, Peptides, Lipid, Hypothalamus, Broiler)

INTRODUCTION

AMPK is the central component of a cellular signaling system that monitors cellular energy change, acting as 'metabolic master switch' to regulate ATP concentrations in the face of stress. AMPK can be activated by allosteric effect of AMP and/or by threonine phosphorylation of one subunit in response to AMPK kinases (Hong et al., 2003; Hawley et al., 2005; Hurley et al., 2005; Hardie, 2006). In some cases, activation of AMPK requires phosphorylation of T-172 by an upstream protein kinase such as LKB1 (Hawley et al., 1996). LKB1 is a serine/threonine protein kinase which was first discovered in the study of Peutz-Jeghers syndrome (Hemminki et al., 1998; Jenne et al., 1998). LKB1 is a complex with two accessory proteins, pseudokinase STRAD (alpha or beta isoforms) and scaffold protein MO25 (alpha and beta isoforms) (Bass et al., 2003; Boudeau et al., 2003). AMPK can be activated in a variety of physiological circumstances, including hypoglycemia (da Silva Xavier et al., 2000), ischemia (Marsin et al., 2000), heat shock (Corton et al., 1994) and exercise (Mu et al., 2001; Musi et al., 2001). AMPK is also affected by several orexigenic and anorexigenic signals in the hypothalamus (e.g. thyroid hormones, ghrelin, insulin and leptin) (Kola et al., 2006). AMPK is widely expressed in the brain, including the areas that control feed intake and neuroendocrine function. Immunostaining revealed that various AMPK isoforms distributed in hypothalamus and the hindbrain (Turnley et al., 1999). In mammals, AMPK exists as a heterotrimeric enzyme complex consisting of one catalytic (alpha) subunit and two regulatory (beta and gamma) subunits (Mitchelhill et al., 1994). There are two known alpha subunit isoforms (alpha-1 and alpha-2, Stapleton et al., 1996), two beta subunit isoforms (beta-1 and beta-2, Stapleton et al., 1997) and three gamma subunit isoforms (gamma-1, gamma-2 and gamma-3, Cheung et al., 2000).

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Previous studies in mammals showed that hypothalamic AMPK integrates nutrient and hormonal signals from both anorexigenic and orexigenic pathways to regulate feed intake (Obici et al., 2003; Andersson et al., 2004; Minokoshi et al., 2004). Minokoshi et al. (2004) reported that fasting resulted in increased hypothalamic AMPK activity, whereas refeeding inhibited it (Minokoshi et al. 2004). AMPK is considered as a major regulator of fatty acid synthesis pathway due to its function to phosphorylate and inactivate key enzymes in fatty acid metabolism such as acetyl-CoA carboxylase (ACC). Pharmacological inhibition of fatty acid synthase (FAS) produces anorexia in mice (Loftus et al., 2000). It is reported that the activation of AMPK in the hypothalamus is associated with activation of mitochondrial enzyme carnitine palmitoyltransferase-1 (CPT1), leading to a decrease in the cellular levels of long-chain acyl-CoAs and increase feed intake (Andersson et al., 2004). The modest reduction of long-chain acyl-CoAs within the arcuate nucleus (ARC) would increase the expression of both agouti-related peptide (AgRP) and neuropeptide Y (NPY) and enhance feed intake (Obici et al., 2003).

There have been numerous reports showing that AMPK is involved in appetite regulation in mammals (Claret et al., 2007; Dzamko et al., 2010). Because feeding behavior and energy homeostasis are basic processes crucial to the survival of all animals (Richards and Proszkowiec-Weglarz, 2007), it is logical to assume that AMPK should be involved in poultry feed intake control. Gene expression of AMPK in chicken embryos has been explored (Proszkowiec-Weglarz and Richards, 2009), but information of its expression in chicks hypothalamus remains scarce. Previous studies in mammals showed that isoform composition of AMPK subunit changes during physiological and pathological conditions (Gregor et al., 2006; Kim et al., 2009). The aim of the present experiment was to investigate if AMPK subunits (α, β and γ) gene expression is modulated by 24 h fasting in meat-type chicks and to determine the interrelationship between AMPK and the mRNA levels of some hypothalamic feeding regulatory neuropeptides (i.e. corticotropin-releasing hormone (CRH), neuropeptide Y (NPY), agouti-related peptide (AgRP), pro-opiomelanocortin (POMC)) and fatty acid synthase (FAS).

MATERIALS AND METHODS

Animals, experimental protocol and sample collection

Male broiler (Arbor Acres) chicks were obtained from a local hatchery at 1 d of age and reared in an environmentally controlled room. The brooding temperature was maintained at 35°C (65% RH) for the first 2 d, and then decreased gradually to 31°C (45% RH) until 10 d. The light regime was 23 L/1 D. All chicks received a starter diet with 21.5% crude protein and 12.33 MJ/kg of metabolizable energy (Zhao et al., 2009). All the birds had free access to feed and water during the rearing period. The study was approved by the University and carried out in accordance with the "Guidelines for Experimental Animal" of Ministry of Science and Technology (Beijing, P. R. China). All the chicks were cared for in accordance with the Guide to the Care and Use of Experimental Animals (Olfert et al., 1993).

Seven-day-old male broiler chicks were allocated into three equal treatments: control, fed *ad libitum* (C); fasted for 24 h (S24); fasted for 24 h and then refed for 24 h (S24R24). At the end of each treatment period, birds were sacrificed by exsanguination (Close, 1997), and then the whole hypothalamus was collected. After snap frozen in liquid nitrogen, the tissue samples were stored at -80°C for RNA extraction.

RNA isolation and analysis

The expression of genes in hypothalamus was quantified using quantitative real-time PCR with SYBR Green I labeling. Total RNA was isolated using the guanidinium isothiocyanate method with Trizol Reagent (Invitrogen,San Diego, CA, USA). The quality of the RNA was tested by electrophoresis on an agarose-gel and the quantity of the RNA was determined with biophotometer (Eppendorf, Germany).

RT reactions (10 μl) consisted of 500 ng total RNA, 5 mmol/L $MgCl_2$, 1 μl RT buffer, 1 mmol/L dNTP, 2.5 U AMV, 0.7 nmol/L oligo d(T) and 10 U Ribonuclease inhibitor (TaKaRa Biotechnology, Co., Ltd. Dalian, P. R. China). Real-time PCR analysis was conducted using the Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Foster, CA, USA). Each RT-reaction served as a template in a 20 μl PCR reaction containing 0.2 μmol/L of each primer and SYBR green master mix (Takara Biotechnology, Co., Ltd. Dalian, P. R. China). Primer-set sequences are described in Table 1. Real-time PCR reactions were performed at 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and 60°C for 34 s. SYBR green fluorescence was detected at the end of each cycle to monitor the amount of PCR product. When calculating the efficiency of qPCR primers, a standard curve was made in 10 fold dilutions, and its slope was used to calculate efficiency.

The relative amount of mRNA for a gene was calculated according to the method of Livak and Schmittgen (Livak and Schmittgen, 2001). The mRNA levels of these genes were normalized to 18s rRNA levels (ΔCT) (Proszkowiec-Weglarz et al., 2006). The ΔCT was calibrated against an average of the control chicks. The linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta CT}$.

Table 1. Gene-specific primers used for the analysis of chick gene expression

Gene	GenBank accession no.	Primer sequences (5'-3')	Orientation	Product size (bp)
18s	AF173612	ATAACGAACGAGACTCTGGCA	Forward	136
		CGGACATCTAAGGGCATCACA	Reverse	
LKB1	NM_001045833	TGAGAGGGATGCTTGAATACGA	Forward	158
		ACTTGTCCTTTGTTTCTGGGC	Reverse	
AMPKa1	DQ302133	CGGAGATAAACAGAAGCACGAG	Forward	266
		CGATTCAGGATCTTCACTGCAAC	Reverse	
AMPKa2	DQ340396	GGGACCTGAAACCAGAGAACG	Forward	215
		ACAGAGGAGGGCATAGAGGATG	Reverse	
AMPKβ1	XM_415278	ATGGTGGACTCCCAGAAGTG	Forward	254
		GAGCACCATCACTCCATCCT	Reverse	
AMPKβ2	BG713266	CTGTCATGGGGAACACCAC	Forward	363
		GGTCCAGGATAGCGACAAAG	Reverse	
AMPKγ1	DQ133597	AGCTGCAGATCGGTACCTACA	Forward	200
		CGTCACGTCCAGGTTGTTGT	Reverse	
AMPKγ2	DQ212708	ATCGGCATTACCTGTTGTGG	Forward	231
		ACCACCAAACGATGAACCTC	Reverse	
NPY	M87294	GAGGCACTACATCAACCTCATCAC	Forward	101
		TGTTTTCTGTGCTTTCCCTCAA	Reverse	
AgRP	NM_001031457	GGAACCGCAGGCATTGTC	Forward	163
		GTAGCAGAAGGCGTTGAAGAA	Reverse	
POMC	NM_001031098	CGCTACGGCGGCTTCA	Forward	88
		TCTTGTAGGCGCTTTTGACGAT	Reverse	
CRH	NM_001123031	CTCCCTGGACCTGACTTTCC	Forward	86
		TGTTGCTGTGGGCTTGCT	Reverse	
Ghrelin	AB075215	CCTTGGGACAGAAACTGCTC	Forward	203
		CACCAATTTCAAAAGGAACG	Reverse	
POMC	NM_001031098	AGAACAGCAAGTGCCAGGAC	Forward	162
		TGCGGAAATGCCTCATCACG	Reverse	
FAS	J03860	CTATCGACACAGCCTGCTCCT	Forward	107
		CAGAATGTTGACCCCTCCTACC	Reverse	
ACCα	NM_205505	AATGGCAGCTTTGGAGGTGT	Forward	136
		TCTGTTTGGGTGGGAGGTG	Reverse	
CPT1	AY675193	GGAGAACCCAAGTGAAAGTAATGAA	Forward	135
		GAAACGACATAAAGGCAGAACAGA	Reverse	
SREBP-1	AY029224	GAGGAAGGCCATCGAGTACA	Forward	392
		GGAAGACAAAGGCACAGAGG	Reverse	
MO25α	XM_422642	GTGGAGATGTCGACGTTTGA	Forward	439
		ATTGTTCATCCTCGGTCCTG	Reverse	
MO25β	NM_0010006272	CTGGAATCTGCTTTCCCATC	Forward	252
		GCAGGATTTTTGTGCGATTT	Reverse	
STRADβ	XM_421938	CTCCATTCATGGCCTATGGT	Forward	558
		TCGTCATTGTGCGTGTCATA	Reverse	

18s = 18s rRNA; LKB1 = Liver kinase B1; AMPK = AMP-activated protein kinase; NPY = Neuropeptide Y; AgRP = Agouti-related peptide; CRH = Corticotropin-releasing hormone; POMC = Pro-opiomelanocortin; FAS = Fatty acid synthase; ACCα = Acetyl-CoA carboxylase α; CPT-1 = Carnitine palmitoyltransferase 1; SREBP-1 = Sterol regulatory element binding protein-1; MO25 = Mouse protein 25; STRAD = STE20 related adaptor protein; STRADβ.

Therefore, all gene transcription results are reported as the n-fold difference relative to the calibrator. Specificity of the amplification product was verified with melt curve.

Statistical analysis

Data are presented as means±SEM. Homogeneity of variances among groups was confirmed using Bartlett's test. All data were subjected to one-way ANOVA analysis testing the main effect of the treatment ((Version 8e, SAS Institute, Cary, NC, USA). When the main effect of treatment was significant, differences between means were assessed by Duncan's multiple range analysis. $p<0.05$ was considered significant.

RESULTS

Expression of AMPK and related genes of hypothalamus during fasting and refeeding

Fasting for 24 h increased the gene expression of AMPKα2, AMPKβ1, AMPKβ2, AMPKγ1, STRADβ and MO25α ($p<0.05$), but showed no significant effects on the gene expression of AMPKα1, AMPKγ2 and LKB1 ($p>0.05$). The gene expression of MO25β was decreased ($p<0.05$) by 24 h fasting. Refeeding for 24 h restored the gene expression of AMPKα2, AMPKγ1 and MO25α to the control levels (Figure 1).

Expression of feed intake regulation peptides of hypothalamus during fasting and refeeding

Fasting for 24 h had no significant ($p>0.05$) effects on gene expression of NPY. However, mRNA levels of POMC, CRH and ghrelin were significantly ($p<0.05$) lower during

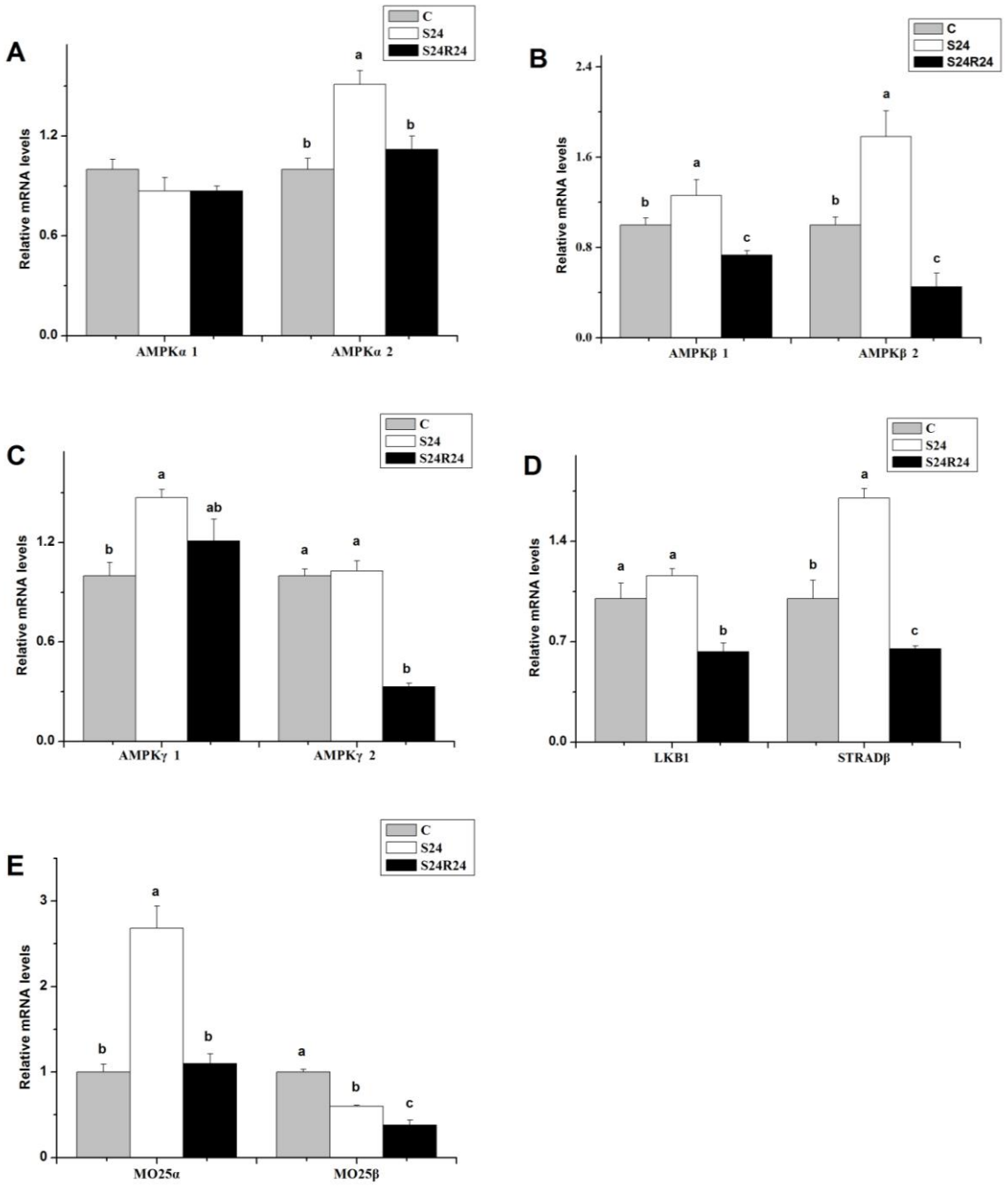

Figure 1. Effect of fasting and refeeding on mRNA levels of AMP-activated protein kinase AMPKα1 (A), AMPKα2 (A), AMPKβ1 (B), AMPKβ2 (B), AMPKγ1 (C), AMPKγ2 (C), LKB1 (D), STRADβ (D), MO25α (E), MO25β (E) in hypothalamus of broiler chicks. Values are means±SEM (n = 4). [a, b] Means with different letters differ significantly ($p<0.05$).

fasting compared to fed control. AgRP gene expression was significantly increased ($p<0.05$) by 24 h fasting. After 24 h of refeeding, mRNA levels of POMC and ghrelin were significantly increased ($p<0.05$). Refeeding decreased mRNA levels of NPY and restored gene expression of AgRP to control levels ($p<0.05$, Figure 2).

Expression of lipid metabolism-related genes in hypothalamus during fasting and refeeding

Fasting for 24 h significantly ($p<0.05$) decreased ACC, FAS, CPT-1 and SREBP-1 mRNA levels compared to *ad libitum* fed controls (Figure 3). Refeeding for 24 h restored ACC, FAS, CPT-1 and SREBP-1 gene expression; in some cases (ACC, FAS, SREBP-1), increased expression levels were significantly above the control levels, which indicated that there was a classical 'overshoot' response ($p<0.05$).

DISCUSSION

Effect of 24 h fasting on gene expression of AMPK subunits in chick's hypothalamus

AMPK has emerged as a nutrient and glucose sensor in the hypothalamus (Momcilovic et al., 2006). It is demonstrated that hypothalamic AMPK activity increases during fasting and decreases during refeeding in mammals (Culmsee, 2001; Minokoshi et al., 2004). AMPK switches on the oxidative metabolism of glucose and fatty acids to generate ATP, while switching off ATP consuming pathways. It achieves this metabolic switching both by direct phosphorylation of metabolic enzymes and via effects on transcription (Hardie and Hawley, 2001; Hardie et al., 2003). However the information of avian AMPK remains obscure, especially its definite roles in avian energy and appetite homeostasis.

In the present study, the expression of AMPK subunits increased after fasting for 24 h, except for α1 and γ2. It has been shown that AMPK subunit genes have tissue-specific expression and both alpha subunit isoforms (alpha-1 and alpha-2) were co-expressed in all tissues examined (Proszkowiec-Weglarz et al., 2006). The current results were inconsistent with some previous reports in chicken liver (Proszkowiec-Weglarz et al., 2009). Whether this

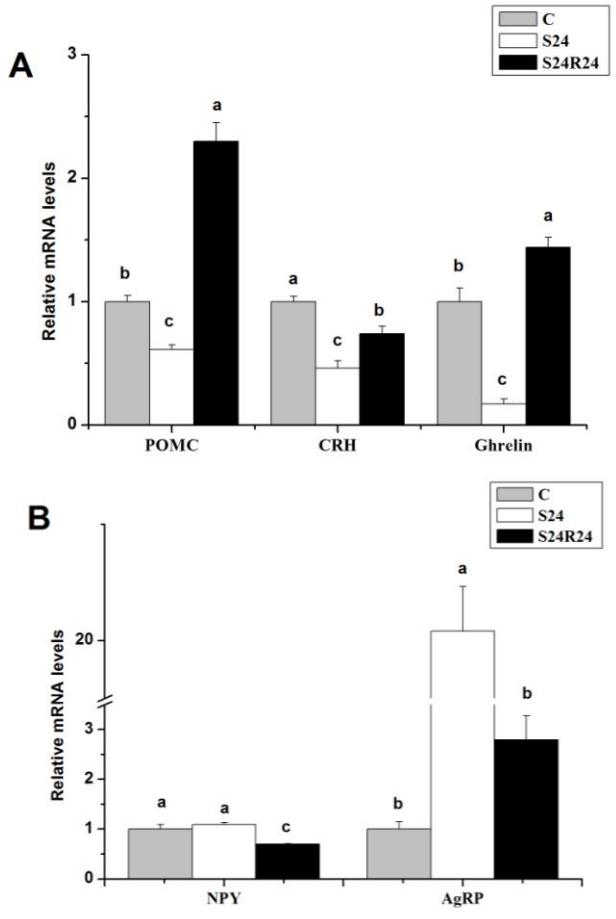

Figure 2. Effect of fasting and refeeding on mRNA levels of POMC (A), CRH (A), ghrelin (A), NPY (B) and AgRP (B) in hypothalamus of broiler chicks. Values are means±SEM (n = 4). [a, b] Means with different letters differ significantly ($p<0.05$).

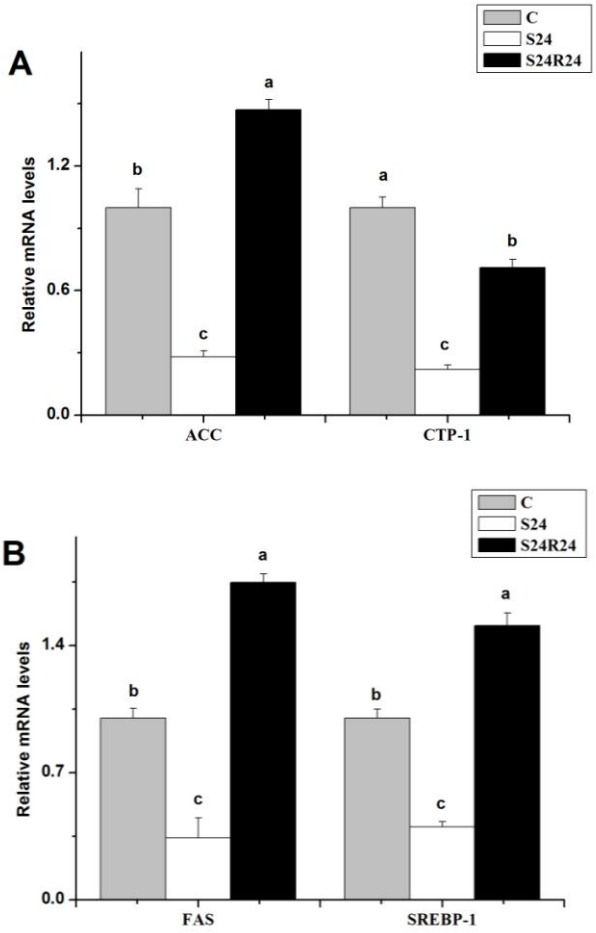

Figure 3. Effect of fasting and refeeding on mRNA levels of ACC (A), CPT-1 (A), FAS (B) and SREBP-1 (B) in hypothalamus of broiler chicks. Values are means±SEM (n = 4). [a,b] Means with different letters differ significantly ($p<0.05$).

difference derived from the tissue specific expression of AMPK needs further investigation. Based on the present results, AMPK α1 was less sensitive to different nutritional conditions than AMPK α2 in the chick's hypothalamus. When chicks were refed, the increased gene expression of AMPK subunits were reduced except AMPK γ1. The integration of these data implied that the different AMPK subunits did not respond to the fasting in the same way, which revealed the complexity of the function of AMPK in the chick's hypothalamus. However, phosphorylation reaction of AMPK protein(s) would be a key of the activation and inactivation of AMPK signaling and the relationships among changes in gene expression of AMPK subunits and its protein phosphorylation during fasting needs further investigation.

LKB1 is one of the AMPK upstream protein kinases (Hawley et al., 1996). Hawley et al. (2003) demonstrated that complexes of LKB1 tumor suppressor, STRAD and MO25 are upstream kinases in the AMPK cascade. In chicken, a functional LKB1/AMPK pathway, similar to the corresponding pathway in mammals has been suggested (Proszkowiec-Weglarz et al., 2006). The hypothalamus of chicken expresses high amount of LKB1, MO25α and both isoforms of STRAD (Proszkowiec-Weglarz et al., 2006). In the present study, the gene expression of MO25β was decreased with 24 h fasting; however, the gene expression of STRADβ and MO25α were increased. The Increased gene expression of STRADβ and MO25α might result in the up-regulation of AMPK α2 in the chick's hypothalamus.

Effect of 24 h fasting on gene expression of appetite regulation peptides in chick's hypothalamus

Multiple neuron populations distributed throughout the brain influence the decision to seek and consume food (Morton et al., 2006). It is known that hypothalamic melanocortin system (HMS) has a crucial rule in feeding regulatory neural circuitry. HMS is composed of two different populations of neurons, one set that expresses neuropeptides Y (NPY) and agouti-related protein (AgRP) and a second set that expresses proopiomelanocortin (POMC), a precursor containing α-melanocyte-stimulating hormone (Richards et al., 2010). In the present study fasting broiler chicks for 24 h did not change mRNA level of NPY in the hypothalamus of broiler chicks, however the mRNA level of AgRP and POMC were increased and decreased respectively (Figure 2).

It is demonstrated that AgRP increase food intake when injected into the brain (Ollmann et al., 1997). Moreover, Takahashi and Cone (2005) discovered that the firing rate of AgRP neurons is elevated in brain slices from food-deprived mice. Hahn et al. (1998) suggested that hypothalamic AgRP neuron constitute a unique cell type that is activated by fasting to stimulate food intake via a simultaneous decrease of melanocortin. In contrast, genetic and pharmacologic evidence suggest that POMC neurons inhibit feeding by releasing α-melanocyte-stimulating hormone, a melanocortin receptor agonist (Tsujii and Bray, 1989; Yaswen et al., 1999). It is suggested that opposite trend of AgRP and POMC gene expression implies that AgRP neurons inhibit POMC neurons in ARC (Cowley et al., 2001; Roseberry et al., 2004).

In the present study, fasting resulted in a decrease in the mRNA level of ghrelin and CRH in the hypothalamus of broiler chicks. It is known that ghrelin acts as orexigenic peptide in mammals when injected centrally and peripherally (Nakazato et al., 2001; Date et al., 2002). In the case of mammals, orexigenic activity of ghrelin is mediated by activation of other orexigenic peptides such as NPY, AgRP and orexin (Toshinai et al., 2003). In neonatal chickens, intracerebroventricular (icv) administration of ghrelin potently inhibits food intake in a dose dependent manner (Khan et al., 2006). Saito et al. (2005) suggested that anorexigenic effects of central ghrelin mediated via CRH in chickens.

The potential relationship between AMPK, appetite regulation peptides and lipid metabolism related genes

All isoforms of AMPK are expressed in neuronal tissues, including these involved in the control of food intake and neuroendocrine function, such as the hypothalamus and the hindbrain (Turnley et al., 1999; Kola, 2008). Pharmacological activation of AMPK in rodent hypothalamus with 5-aminoimidazole-4-carboxamide riboside (AICAR) causes an increase in food intake (Xue and Kahn, 2006). In line with this, it has been also recently reported that AMPKβ1 KO mice reduce food intake, either under low fat (LFD) or HFD (Dzamko, 2010). Deletion of AMPKα2 in AgRP neurons led to the development of an age-dependent lean phenotype (Claret et al., 2007; Lim et al., 2010). In the present study, the enhanced gene expression of α2, β1, β2 and γ1 isoforms of AMPK was accompanied by a reduction in the mRNA levels of POMC and ghrelin and an enhancement of the AgRP mRNA levels in the hypothalamus of broiler chicks after 24 h fasting. Based on these results, it was speculated that AMPK might be involved in the appetite regulation in the hypothalamus of the chicks. AMPK might have enhanced the appetite of chicks via increasing the orexigenic gene expression of AgRP and decreasing the anorexigenic gene expression of POMC in chicks with 24 h fasting.

The level of metabolic flux through the fatty acid biosynthetic pathway in hypothalamic neurons as regulated by AMPK determines the levels of two key metabolites, malonyl-CoA, and long chain fatty acyl-CoA, which in turn lead to changes in feed intake and energy expenditure by altering the expression of orexigenic and anorexigenic

neuropeptides in melanocortin system neurons (Lam et al., 2005; Lane et al., 2005; He et al., 2006). AMPK can regulate the transcription and expression of FAS and ACCα in hypothalamus of mammals and avian species (Xue and Kahn, 2006; Xu et al., 2011a; Xu et al., 2011b). The AMPK inhibits the activity of ACC, the rate-limiting enzyme involved in the production of malonyl-CoA used for fatty acyl-CoA biosynthesis, and causes a reduction in this reaction, which stimulates CPT1 and reduces the flux of substrates in the fatty acid anabolic pathway (Carling et al., 2008; Lage et al., 2008). In this study, although we anticipated an increase in the mRNA level of CPT1, however, 24 h fasting significantly decreased CPT1 gene expression in the hypothalamus of broiler chicks. The underlying mechanism needs further investigation.

In our experiment, the mRNA levels of FAS and SREBP-1 were reduced after 24 h fasting and refeeding caused an overshooting of FAS and SREBP-1 gene expression. FAS inhibitor reduces food intake in mammals and chickens (Dridi et al., 2006). Feed restriction decreased gene expression of FAS in an AMPK-dependent manner in mammals (López et al., 2008) and chickens (Dridi et al., 2006; Proszkowiec-Weglarz et al., 2006). AMPK can directly decrease FAS gene transcription via regulating the gene expression of SREBP-1 in mammals and avian (Foretz et al., 2005; Shaw et al., 2005; Proszkowiec-Weglarz et al., 2009). Bennett et al. (2008) recently reported that the lipogenic enzyme gene expression 'overshoot' in response to refeeding with a high carbohydrate diet in mice following a fast, which was attributable to enhanced binding of SREBP-1c to SRE sites located in lipogenic enzyme target gene promoters, as well as increased co-regulatory protein recruitment. In line with previous reports, the present findings revealed that the AMPK/SREBP-1/FAS signal pathway might be involved in the 24 h fasting induced appetite regulation in chicks.

In conclusion, 24 h fasting altered gene expression of AMPK subunits, appetite regulation peptides and lipometabolism related factors in chick's hypothalamus; the hypothalamic FAS signal pathway might be involved in the AMPK regulated energy homeostasis and/or appetite regulation in poultry.

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