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Characterization of the AMP-activated protein kinase pathway in chickens☆

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Abstract

In mammals, AMP-activated protein kinase (AMPK) is involved in the regulation of cellular energy homeostasis and, on the whole animal level, in regulating energy balance and food intake. Because the chicken is a valuable experimental animal model and considering that a first draft of the chicken genome sequence has recently been completed, we were interested in verifying the genetic basis for the LKB1/AMPK pathway in chickens. We identified distinct gene homologues for AMPK alpha, beta and gamma subunits and for LKB1, MO25 and STRAD. Analysis of gene expression by RT-PCR showed that liver, brain, kidney, spleen, pancreas, duodenum, abdominal fat and hypothalamus from 3 wk-old broiler chickens preferentially expressed AMPK alpha-1, beta-2 and gamma-1 subunit isoforms. Heart predominantly expressed alpha-2, beta-2 and gamma-1, whereas skeletal muscle expressed alpha-2, beta-2 and gamma-3 preferentially. Moreover, the AMPK gamma-3 gene was only expressed in heart and skeletal muscle. Genes encoding LKB1, MO25 alpha, MO25 beta, and STRAD beta were expressed in all examined tissues, whereas STRAD alpha was expressed exclusively in brain, hypothalamus, heart and skeletal muscle. Alterations in energy status (fasting and refeeding) produced little significant change in AMPK subunit gene transcription. We also determined the level of phosphorylated (active) AMPK in different tissues and in different states of energy balance. Immunocytochemical analysis of the chicken hypothalamus showed that activated AMPK was present in hypothalamic nuclei involved in regulation of food intake and energy balance. Together, these findings suggest a functional LKB1/AMPK pathway exists in chickens similar to that observed in mammals.
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Keywords: AMPK; Chicken; Energy balance; Gene expression; Hypothalamus; LKB1; MO25; Protein kinase; Phosphorylation; STRAD

1. Introduction

In mammals, AMP-activated protein kinase (AMPK) exists as a heterotrimeric enzyme complex consisting of one catalytic (alpha) subunit and two regulatory (beta and gamma) subunits (Mitchelhill et al., 1994; Stapleton et al., 1994). There are two known alpha subunit isoforms, designated 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) (Gao et al., 1996; Cheung et al., 2000). Alpha subunits contain a kinase domain at the N-terminus, an internal regulatory domain with an auto-inhibitory region that inhibits kinase activity in the absence of AMP and a beta–gamma binding domain at the C-terminus (Crute et al., 1998; Kemp et al., 2003). The core of each beta subunit contains a glycogen binding domain (GBD), whereas the C-terminus is responsible for binding the alpha and gamma subunits (Thornton et al., 1998; Hudson et al., 2003; Kemp et al., 2003). Gamma subunits contain four repeats of a structural motif classified as a cystathionine β-synthase (CBS) domain (Bateman, 1997). Two CBS domains form a functional unit recently designated as a "Bateman domain" (Kemp, 2004). Bateman domains function as cooperative regulatory AMP- and ATP-binding sites within the AMPK complex (Scott et al., 2004).

In the absence of AMP, the AMPK complex exists in an inactive conformation in which alpha and gamma subunits do not interact (Cheung et al., 2000). In the active state, association between the catalytic cleft and the auto-inhibitory region on the alpha subunit is disrupted by the interaction of the latter

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with one or more CBS domains on the gamma subunit. This interaction between the alpha subunit auto-inhibitory region and the gamma subunit Bateman domain is stabilized by binding of AMP (Cheung et al., 2000). Binding of AMP to the Bateman domain results in direct allosteric activation of AMPK through phosphorylation of threonine-172 (T-172, located within the activation loop of the alpha subunit) and inhibition of its dephosphorylation by protein phosphatases (Davies et al., 1995; Hawley et al., 1995; Hardie, 2004b). AMPK is activated by a variety of metabolic stresses that not only lower cellular energy levels resulting in a rise in the AMP/ATP ratio, but also alter the cellular redox potential resulting in an increase in the NAD/NADH ratio. AMPK can also be activated by a nucleotide-independent mechanism that has not been well characterized (Hardie et al., 1998; Rafaeloff-Phail et al., 2004). In all 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 as a result of a gene mutation leading to Peutz–Jeghers syndrome (Hemminki et al., 1998; Jenne et al., 1998). LKB1 exists as a complex with two accessory proteins, pseudokinase STRAD (alpha or beta isoform) and scaffold protein MO25 (alpha or beta isoform) (Bass et al., 2003; Boudeau et al., 2003).

Once activated, AMPK increases cellular energy supply by switching on ATP-generating pathways and decreases energy demand by switching off ATP-utilizing pathways. This leads to: 1) inhibition of fatty acid and cholesterol synthesis in liver and lypolysis in adipocytes as a result of phosphorylation of acetyl-CoA carboxylase-1 (ACC-1), HMG-CoA reductase and hormone sensitive lipase (Gillespie and Hardie, 1992; Corton et al., 1995); 2) inhibition of fatty acid synthesis via reduced transcription of genes encoding ACC-1 and fatty acid synthase (FAS) (Woods et al., 2000); 3) stimulation of fatty acid uptake in muscle (Luiken et al., 2003); and 4) activation of fatty acid oxidation in muscle by phosphorylation of ACC-2 (Merrill et al., 1997; Hardie, 2004a,b). Moreover, AMPK acts not only through direct phosphorylation of metabolic enzymes, but also through effects on gene expression via several important transcription factors, including sterol response element binding protein 1c (Zhou et al., 2001), hepatocyte nuclear factor-4α (Leclerc et al., 2001; Hong et al., 2003), CAAT/enhancer binding protein, peroxisome proliferator-activated receptor-γ (Habinowski and Witters, 2001) and peroxisome proliferator-activated receptor-α (Bronner et al., 2004; Hardie, 2004a). There are also indications that AMPK links the sensing of intracellular energy level with the regulation of protein synthesis (Jones et al., 2005). Thus, AMPK serves as a "fuel gauge" that responds to fluctuations in cellular energy level, as well as, to the levels of specific extracellular nutrients such as glucose and fatty acids and hormones such as leptin, adiponectin and ghrelin (Carling, 2005). Recent studies suggest that AMPK not only regulates the energy level within the cell, but may also play a role in regulating whole-body energy metabolism by responding to circulating hormones such as leptin and adiponectin and by controlling food intake (Yamauchi et al., 2002; Andersson et al., 2004; Minokoshi et al., 2004).

Although gene homologues of AMPK subunits have been identified in a number of non-mammalian organisms including rye, barley, tobacco, yeast, Drosophila and Caenorhabditis elegans (Gao et al., 1996), to date there has been no investigation of the AMPK pathway in birds. Because the chicken is a valuable experimental animal model and considering that a first draft of the chicken genome sequence has been recently completed (Wallis et al., 2004), the aim of the present study was to identify and characterize the LKB1/AMPK pathway in chickens.

2. Materials and methods

2.1. Animals

All animal studies were conducted according to research protocols approved by the Beltsville Animal Care and Use Committee. Day-old male broiler chicks (*Gallus gallus domesticus*) were purchased from Hubbard-ISA (Duluth, GA, USA) and grown until 3 weeks of age. A standard commercial diet and water were provided ad libitum.

2.2. Experiment I

Samples ($n=6$) of liver, brain, heart, kidney, spleen, duodenum, skeletal muscle, abdominal fat, pancreas and hypothalamus tissue were collected from 3 wk-old broiler chickens fed ad libitum and snap frozen in liquid nitrogen prior to RNA or protein isolation.

2.3. Experiment II

Thirty 3 wk-old broiler chickens were divided into five equal treatment groups: control, fed ad libitum (C); fasted for 24 h (S24); fasted for 24 h and then refed for 24 h (SR24); fasted for 48 h (S48); and fasted for 48 h and then refed for 24 h (SR48). At the end of the treatment period, tissue samples described in Experiment I were collected and snap frozen in liquid nitrogen prior to RNA and protein isolation.

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription (RT) reactions (20 μL) consisted of: 1.0 μg total RNA, 50 units Superscript III reverse transcriptase (Invitrogen), 40 units of an RNAse inhibitor (Invitrogen), 0.5 mM dNTPs, and 100 ng of random hexamer primers. Polymerase chain reaction (PCR) was performed in 25 μL reactions containing: 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.0 unit of Platinum Taq DNA polymerase (Invitrogen), 0.2 mM dNTPs, 2.0 mM Mg^{2+}, 10 pmol of each gene specific primer (see Table 1 for primer sequence), 5 pmol each of an appropriate mixture of primers:competimers specific for 18S rRNA (QuantumRNA Universal 18S Standards kit, Ambion, Inc., Austin, TX, USA),

Table 1
Gene-specific primers for the analysis of chicken AMPK pathway gene expression by RT-PCR

Gene	Accession number[a]	Primer sequence (5′→3′)	Orientation	Product size (bp)
LKB1	XM_418227	ATTCCAGCCACCAGAAATTG	Forward	395
		CCTCATTGTAGCCATGCAGA	Reverse	
MO25 α	XM_422642	GTGGAGATGTCGACGTTTGA	Forward	439
		ATTGTTCATCCTCGGTCCTG	Reverse	
MO25 β	NM_0010006272	CTGGAATCTGCTTTCCCATC	Forward	252
		GCAGGATTTTTGTGCGATTT	Reverse	
STRAD α	BX929431	TCCGGGTGACTCTAGGAGAA	Forward	506
		TCAGGATATGGCTGGCTTTC	Reverse	
STRAD β	XM_421938	CTCCATTCATGGCCTATGGT	Forward	558
		TCGTCATTGTGCGTGTCATA	Reverse	
AMPK α1	XM_424772	AAGGTTGGCAAGCATGAGTT	Forward	492
		TTCTGGGCCTGCATATAACC	Reverse	
AMPK α2	XM_426666	AGCACGCCAACAGACTTCTT	Forward	399
		ATCATCAAAGGGCAAAGTGC	Reverse	
AMPK β1	XM_415278	ATGGTGGACTCCCAGAAGTG	Forward	254
		GAGCACCATCACTCCATCCT	Reverse	
AMPK β2	BG713266	CTGTCATGGGGAACACCAC	Forward	363
		GGTCCAGGATAGCGACAAAG	Reverse	
AMPK γ1	DQ133597	AGCTGCAGATCGGTACCTACA	Forward	200
		CGTCACGTCCAGGTTGTTGT	Reverse	
AMPK γ2	DQ212708	ATCGGCATTACCTGTTGTGG	Forward	231
		ACCACCAAACGATGAACCTC	Reverse	
AMPK γ3	DQ079814	GGATGCTCACCATCACTGAC	Forward	402
		CCAGAGCAGCATACACAGGA	Reverse	

All primers used for expression analyses were designed using the Primer3 program (Rozen and Skaletsky, 2000) in regions of the gene for which sequence had been previously verified by molecular cloning and direct sequencing of PCR generated fragments.
[a] Reference chicken gene sequences that contain the corresponding PCR products listed.

and 1 μL of the RT reaction. Thermal cycling parameters were 1 cycle 94 °C for 2 min, followed by 30 cycles, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 8 min. AMPK subunits, MO25 and STRAD, were co-amplified with 18S rRNA in a multiplex PCR format. Negative RT controls were run to ensure PCR accuracy and specificity. PCR products for LKB1, STRAD, MO25 and AMPK subunits were isolated using a GenElute PCR clean-up kit (Sigma Chemical Co, St. Louis, MO, USA) and their sequences were confirmed by bi-directional automated fluorescent DNA sequencing (CEQ 8000XL, Beckman Coulter, Inc., Fullerton, CA, USA).

2.5. Capillary electrophoresis with laser induced fluorescence detection (CE-LIF)

Relative quantitation of PCR products was accomplished using CE-LIF as described previously (Richards and Poch, 2002). Aliquots (2 μL) of RT-PCR samples were first diluted 1 : 100 with deionized water. A P/ACE MDQ (Beckman Coulter) equipped with an argon ion LIF detector was used. Capillaries were 75 μm I.D.×32 cm μSIL-DNA (Agilent Technologies, Palo Alto, CA, USA). EnhanCE™ dye (Beckman Coulter) was added to the DNA separation buffer (Sigma) to a final concentration of 0.5 μg/mL. Samples were loaded by electrokinetic injection at 3.5 kV for 5 s and run in reverse polarity at 8.1 kV for 3.5 min. P/ACE MDQ software (Beckman Coulter) was used to calculate peak areas for the PCR amplicons separated by CE.

2.6. Quantitation of gene expression

The levels of LKB1, STRAD, MO25 and AMPK subunit gene expression were determined as the ratio of integrated peak area for each PCR product relative to that of the co-amplified 18S rRNA internal standard. Values are presented as the mean±SE of individual expression ratio determinations ($n=6$ birds).

2.7. Protein isolation

Tissues were homogenized in lysis buffer (50 mM Tris–HCl, pH 7.5; 250 mM sucrose, 5 mM sodium pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 0.1 mM benzamidine, 50 μg/mL of leupeptin and soybean trypsin inhibitor; Sigma). Homogenates were centrifuged at 14,000 ×*g* for 30 min at 4 °C. The supernatant was collected and proteins were precipitated using polyethylene glycol 8000 (PEG 8000, Sigma) as follows: 0.1 mL of a 25% (w/v) PEG 8000 stock solution was added to 0.9 mL of the supernatant to yield a PEG concentration of 2.5% (w/v) and the sample was centrifuged for 3 min (18,000 ×*g*, 4 °C). After centrifugation 1 part of the 25% PEG 8000 stock solution was added to 9 parts of supernatant to bring the PEG concentration to 4.75% (w/v). The sample was centrifuged for 3 min (18,000 ×*g*, 4 °C) and the final pellet was resuspended in 150–300 μL of lysis buffer. Protein concentration was estimated by the Bradford method (Bio-Rad Protein Assay kit II; Bio-Rad Laboratories, Hercules, CA, USA) and samples were snap frozen in liquid nitrogen.

2.8. Western blot analysis

Protein extracts (15–25 μg) were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions on 12% gels according to the method of Laemmli (1970). Separated proteins were then transferred to PVDF membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA) using a semi-dry electro-blotting system (Bio-Rad) for 1.5 h at 16–25 V in Tris–glycine buffer containing 20% methanol. Membranes were blocked with BSA (Sigma) for 16–24 h and incubated overnight with diluted (1 : 1000) primary antibody against phosphorylated AMPK alpha isoforms (Phospho-AMPK-α/Thr172 antibody, Cell Signaling Technology, Inc. Beverly, MA, USA) followed by a horse radish peroxidase conjugated secondary antibody against rabbit IgG (1 : 10,000, Amersham Biosciences, Piscataway, NY, USA). Immunoreactive bands were visualized with ECL Plus Western blotting detection reagents (Amersham). The membranes were scanned and the band intensity was quantified using a Typhoon 9400 variable mode imager (Amersham) and Imagequant software (Molecular Dynamics, Inc., Sunnyvale, CA, USA). Dilutions and all incubations with antibody were performed in Tris–saline buffers containing 0.05% Tween-20 and 0.5% BSA (Sigma).

2.9. Slot blot analysis

An AMPK standard curve was prepared with aliquots (7.8 to 125 mU)[1] of AMPK (partially purified from rat liver; Upstate, Lake Placid, NY, USA) diluted in water to a final volume of 200 μL and transferred to 0.45 μm nitrocellulose membranes pre-soaked in Tris–saline buffer using a slot blotting device under gentle vacuum (Bio-Rad). The membrane blocking, antibody incubation procedures, visualization and quantification of the bands were the same as described for Western blot analysis. Background staining was estimated using blank samples without AMPK. Band intensity (arbitrary units) was plotted against the amount of active AMPK (mU) loaded per lane and linear regression analysis was used to analyze the data.

2.10. Immunocytochemistry

Chickens fed ad libitum ($n=4$) were anesthetized with sodium pentobarbital and perfused with heparinized physiological saline followed by 4% paraformaldehyde. Brains were removed from the cranium, dehydrated, cleared in xylene and embedded in paraffin. Sections (6 μm) containing the hypothalamus were mounted on glass slides. After deparaffinization in xylene the sections were rehydrated in decreasing concentrations of ethanol/water and transferred to a Tris (10 mM, pH 7.5)–saline (150 mM) solution. To block the activity of endogenous peroxidase and non-specific binding, sections were incubated for 30 min in methanolic H_2O_2 followed by 3% normal goat serum for 1 h. The slides were then incubated overnight (humidified chamber, 4 °C) with diluted (1 : 500) primary antibody against phosphorylated AMPK alpha isoforms (Cell Signaling Technology, Inc.). Signal was visualized using the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) with diaminobenzidine (Sigma) as the substrate and the sections were counter-stained with Carrazi's alum hematoxylin. Slides incubated without primary antibody were used as a negative control. Each section was photographed at full illumination using 20× or 40× objectives on an Olympus BX-40 microscope with an Olympus DP-70 digital camera.

2.11. Statistical analysis

Gene and protein expression data were subjected to analysis of variance (ANOVA) using the general linear models (GLM) procedure of SAS software (The SAS System for Windows, v. 8.2; SAS Institute Cary, NC, USA). The Duncan's multiple range test option of the GLM procedure for SAS was used to determine significance of mean differences. Significance was set at $P<0.05$.

3. Results

3.1. Characterization and localization of AMPK pathway genes

Portions of chicken LKB1, STRAD, MO25 and AMPK subunit gene homologues were identified using a primer-directed RT-PCR approach. A Blast search of the chicken genome (http://www.ensembl.org/Gallus_gallus/) was performed for each sequenced PCR product so that they could be verified, aligned with known genomic sequence (including any predicted gene transcripts) and assigned to specific chromosome locations (Table 2). Like mammals, chickens have two isoforms of STRAD, MO25 and AMPK alpha and beta subunits. Three

Table 2
Chromosomal location of LKB1, MO25, STRAD and AMPK subunit genes

	Chromosome	
	Human[a]	Chicken
LKB1	19	28
STRAD subunits		
α	17	27
β	1 or 7	7
MO25 subunits		
α	2	9
β	13	1
AMPK subunits		
α 1	5	Z[b]
α 2	1	8
β 1	12	15
β 2	1	?
γ 1	12	?
γ 2	7	2
γ 3	2	7

[a] Human chromosome assignments were made according to Carling (2004).
[b] Z is a sex chromosome in birds with females being heterogametic ZW and males ZZ.

[1] One unit of AMPK activity is defined as 1 nmol phosphate incorporated into 100 μM SAMS peptide substrate (HMRSAMSGLHLVKRR) per minute at 30 °C with a final ATP concentration of 100 μM (Upstate, Lake Placid, NY, USA).

Table 3
Amino acid sequence comparisons between chicken and human LKB1 and associated proteins STRAD and MO25

	Chicken	Human	Chicken vs. human	
LKB1 kinase domain	–	–	92%	
STRAD (kinase domain)	α vs. β	α vs. β	α	β
	81%	81%	83%	95%
MO25	α vs. β	α vs. β	α	β
	69%	83%	81%	96%

The Genbank accession numbers for human LKB1, STRAD α and β and MO25 α and β subunits were: NP_000446, AAG48269, AAM19143, NP_057373 and CAC37735, respectively.

isoforms of the AMPK gamma subunit were identified. However, the chromosomal locations of the AMPK beta-2 and gamma-1 subunits are presently unknown (Table 2). Using information derived from the chicken genome, EST sequence databases and molecular cloning techniques, we have successfully begun sequencing larger portions of the gamma-1, gamma-2 and gamma-3 AMPK subunit genes (cDNAs) including full coding regions, portions of the 5′- and 3′-untranslated regions and specific variants that arise from alternative splicing of primary gene transcripts (GenBank Accession Nos. DQ133597, DQ133598, DQ079814, DQ079815 and DQ212708 DQ212709 DQ212710 DQ212711). This sequence information offers new insight into chicken AMPK gamma subunit gene structure and function.

Chicken LKB1, STRAD and MO25 showed very high homology, ranging from 81% to 96%, at the amino acid level in comparison with the corresponding human sequence (Table 3). Similarly, chicken AMPK subunits showed very high homology at the amino acid level compared to human AMPK subunits (Table 4). For the kinase domain of AMPK alpha subunits, the similarity between human and chicken sequence was 99%. The similarity of the glycogen binding and the interacting domains of AMPK beta subunits ranged from 83% to 95% and 95% to 96% compared to respective human sequence. The homology of the gamma subunit CBS domains in chickens compared to humans ranged from 68% to 100% (Table 4).

3.2. Expression of AMPK pathway genes

The *LKB1* gene was expressed in all tissues examined (Fig. 1). The highest levels of expression for this gene were observed in duodenum and skeletal muscle while the lowest levels were in liver and pancreas. The beta isoform of STRAD was widely expressed in chicken tissues, whereas the alpha isoform was exclusively expressed in brain, hypothalamus, heart and skeletal muscle (Fig. 2). Alpha and beta subunits of MO25 were expressed in all tissues with the alpha isoform being expressed preferentially. The hypothalamus displayed the highest level of expression of MO25 alpha (Fig. 3).

AMPK subunit genes showed tissue-specific expression. Both alpha subunit isoforms (alpha-1 and alpha-2) were co-expressed in all tissues examined. The alpha-2 isoform was expressed preferentially in heart and skeletal muscle (Fig. 4). The highest levels of alpha-1 and alpha-2 gene expression were observed in abdominal fat and skeletal muscle, respectively. Beta-2 was preferentially expressed with the highest levels in skeletal muscle and abdominal fat (Fig. 5). Fig. 6 presents expression levels of the three different gamma subunit genes. Gamma-1 and gamma-2 were expressed in all tissues, whereas gamma-3 was expressed exclusively in heart and skeletal muscle. Gamma-1 was preferentially expressed in liver, heart, kidney, spleen, duodenum and pancreas. There were no significant differences ($P>0.05$) in the level of expression of gamma-1 and gamma-2 in brain, abdominal fat and hypothalamus (Fig. 6). The gamma-3 gene was preferentially expressed in skeletal muscle.

3.3. Expression of AMPK subunit genes in response to fasting and refeeding

Fasting and refeeding generally had no major effects on expression of AMPK subunit genes in different tissues examined (data not shown). However, small changes were seen in skeletal muscle for the expression of alpha-1 and alpha-2 isoform genes (Fig. 7). Alpha-2 expression declined significantly

Table 4
Amino acid sequence comparisons of chicken and human AMPK subunits

		Chicken			Human			Chicken vs. human		
α subunits		α1 vs. α2			α1 vs. α2			α1		α2
	Kinase domain	90%			90%			99%		99%
β subunits		β1 vs. β2			β1 vs. β2			β1		β2
	Glycogen binding domain	81%			81%			83%		95%
	Interacting domain	93%			88%			96%		95%
γ subunits		γ1 vs. γ2	γ1 vs. γ3	γ2 vs. γ3	γ1 vs. γ2	γ1 vs. γ3	γ2 vs. γ3	γ1	γ2	γ3
	CBS domains	75–96%	59–88%	53–89%	68–94%	61–86%	61–86%	90–100%	88–100%	68–88%

The GenBank accession numbers for human α1, α2, β1, β2, γ1, γ2 and γ3 AMPK subunit sequences were: NP_006242, NP_006243, NP_006244, NP_005390, NP_002724, NP_057287 and NP_059127, respectively.

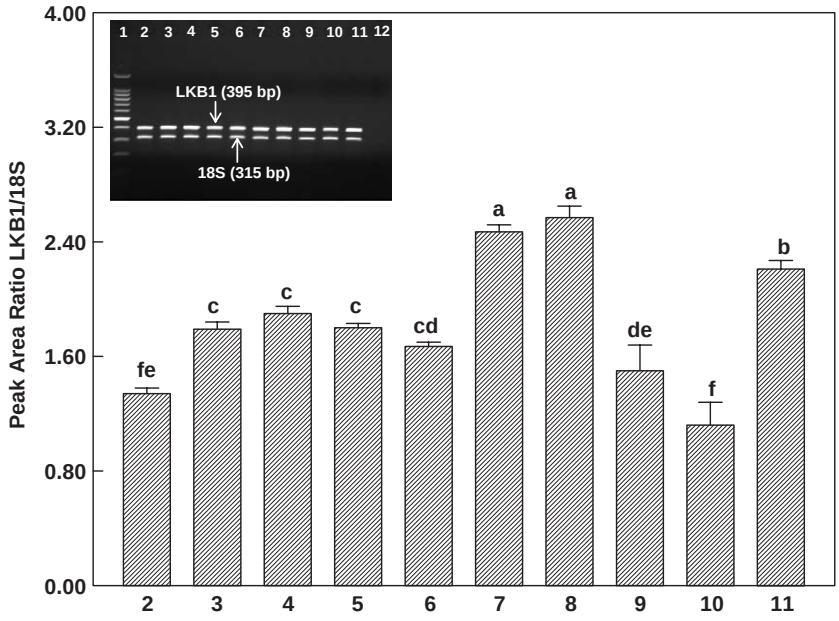

Fig. 1. Expression of the *LKB1* gene in different tissues from 3 wk-old broiler chickens. RT-PCR and CE-LIF were used to quantify the level of expression relative to an 18S rRNA internal standard. Expression ratio (LKB1/18S) values represent the mean±SE (n=6 birds). Different letters above each bar denote statistically significant (P<0.05) differences for mean comparisons. The inset figure presents a typical separation of PCR reactions containing LKB1 and 18S rRNA amplicons on a 1.5% agarose gel stained with ethidium bromide. Samples are identified as follows: (1) 100 bp ladder, (2) liver, (3) brain, (4) heart, (5) kidney, (6) spleen, (7) duodenum, (8) skeletal muscle, (9) abdominal fat, (10) pancreas, (11) hypothalamus and (12) negative PCR control.

(P<0.05) in response to fasting and subsequent refeeding increased expression above control levels. Conversely, alpha-1 expression increased in response to fasting, reaching significance (P<0.05) by 48 h. Subsequent refeeding reduced expression back to control levels.

3.4. Activity of AMPK and its response during fasting and refeeding

To study whether a prepared AMPK standard could be used to associate AMPK enzyme activity and alpha subunit

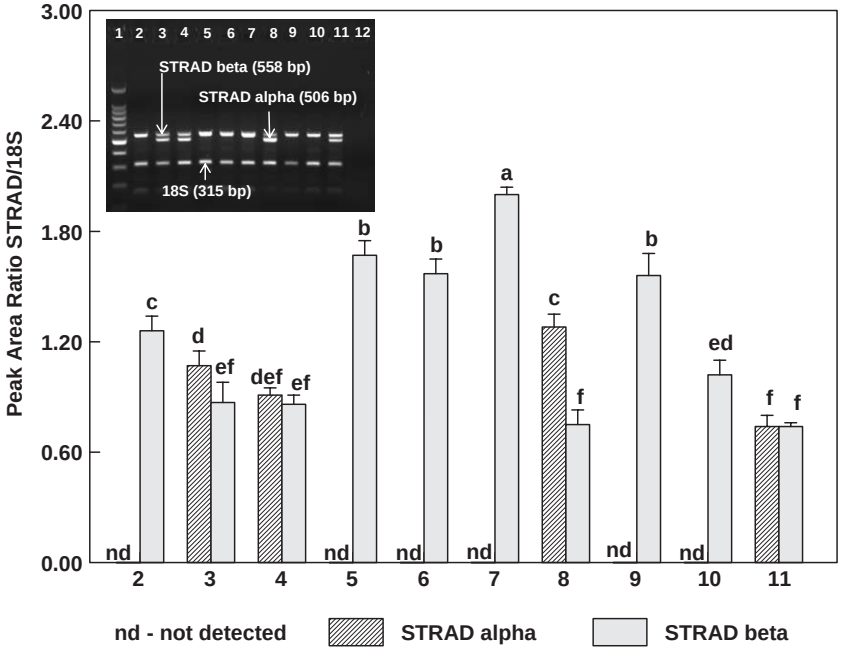

Fig. 2. Expression of *STRAD* genes in different tissues from 3 wk-old broiler chickens. RT-PCR and CE-LIF were used to quantify the level of expression relative to an 18S rRNA internal standard. Expression ratio (STRAD/18S) values represent the mean±SE (n=6 birds). Different letters above each bar denote statistically significant (P<0.05) differences for mean comparisons. The inset figure presents a typical separation of PCR reactions containing STRAD alpha, STRAD beta and 18S rRNA amplicons on a 1.5% agarose gel stained with ethidium bromide. Samples are identified in the legend to Fig. 1.

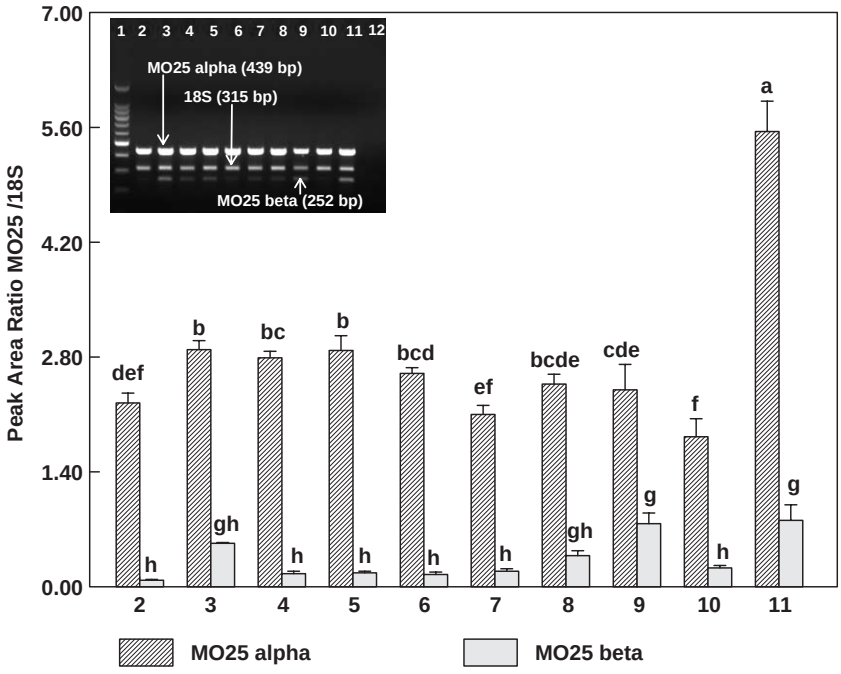

Fig. 3. Expression of *MO25* genes in different tissues from 3 wk-old broiler chickens. RT-PCR and CE-LIF were used to quantify the level of expression relative to an 18S rRNA internal standard. Expression ratio (MO25/18S) values represent the mean±SE (n=6 birds). Different letters above each bar denote statistically significant (P<0.05) differences for mean comparisons. The inset figure presents a typical separation of PCR reactions containing MO25 alpha, MO25 beta and 18S rRNA amplicons on a 1.5% agarose gel stained with ethidium bromide. Samples are identified in the legend to Fig. 1.

phosphorylation in tissues by immunoblot analysis, a standard curve was constructed using 7.8 to 125 mU of active AMPK (Fig. 8). Band intensity showed a significant linear response (r^2=0.996) between 7.8 and 125 mU of added active AMPK. This clearly demonstrated a direct linear relationship between level of AMPK phosphorylation and enzyme activity.

Western blot analysis using an antibody specific for phosphorylated AMPK alpha subunits showed statistically

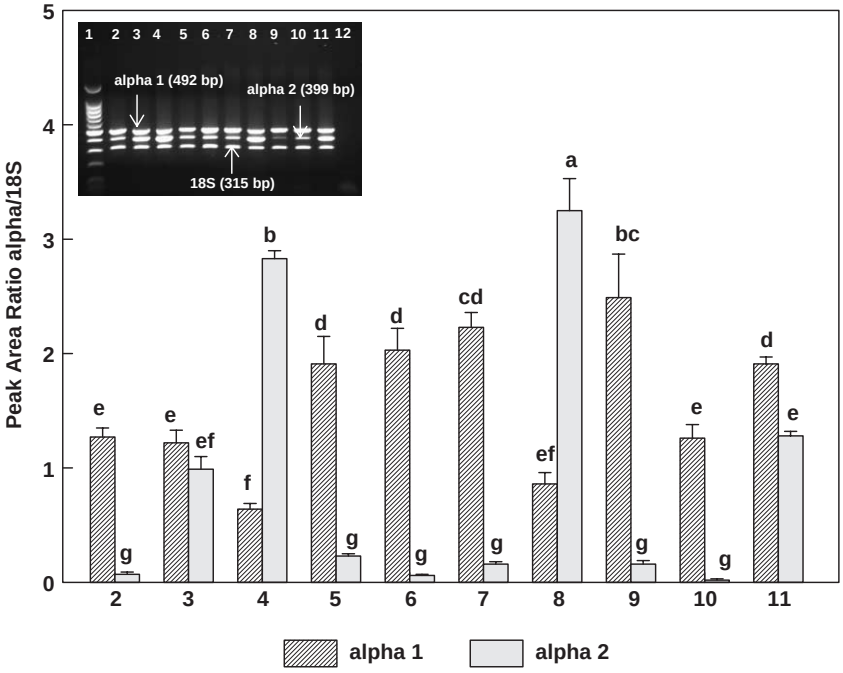

Fig. 4. Expression of the AMPK alpha subunit genes in different tissues from 3 wk-old broiler chickens. RT-PCR and CE-LIF were used to quantify the level of expression relative to an 18S rRNA internal standard. Expression ratio (alpha/18S) values represent the mean±SE (n=6 birds). Different letters above each bar denote statistically significant (P<0.05) differences for mean comparisons. The inset figure presents a typical separation of PCR reactions containing AMPK alpha-1, AMPK alpha-2 and 18S rRNA amplicons on a 1.5% agarose gel stained with ethidium bromide. Samples are identified in the legend to Fig. 1.

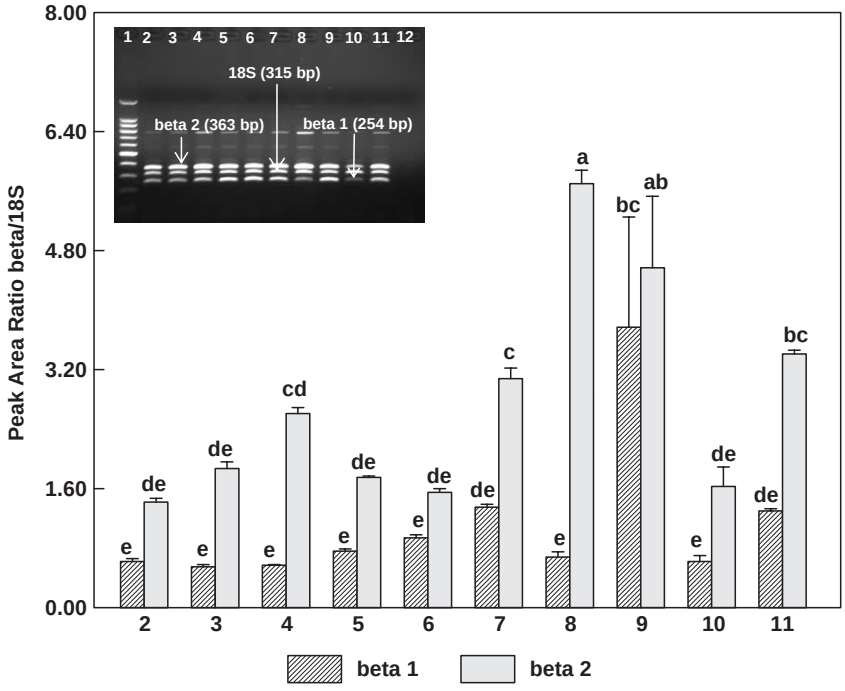

Fig. 5. Expression of AMPK beta subunit genes in different tissues from 3 wk-old broiler chickens. RT-PCR and CE-LIF were used to quantify the level of expression relative to an 18S rRNA internal standard. Expression ratio (beta/18S) values represent the mean±SE ($n=6$ birds). Different letters above each bar denote statistically significant ($P<0.05$) differences for mean comparisons. The inset figure presents a typical separation of PCR reactions containing AMPK beta-1, AMPK beta-2 and 18S rRNA amplicons on a 1.5% agarose gel stained with ethidium bromide. Samples are identified in the legend to Fig. 1.

significant differences ($P<0.05$) in the level of phosphorylated AMPK among different tissues from chickens fed ad libitum (Fig. 9). The lowest level of phosphorylated AMPK was observed in abdominal fat, whereas the highest levels were found in liver, heart and spleen.

Fasting for 24 or 48 h (S24, S48) increased phosphorylated AMPK; however, the increase was only statistically significant ($P<0.05$) in skeletal muscle from chickens fasted for 48 h (Fig. 10). After 24 h of refeeding (SR24 or SR48), the level of phosphorylated AMPK did not

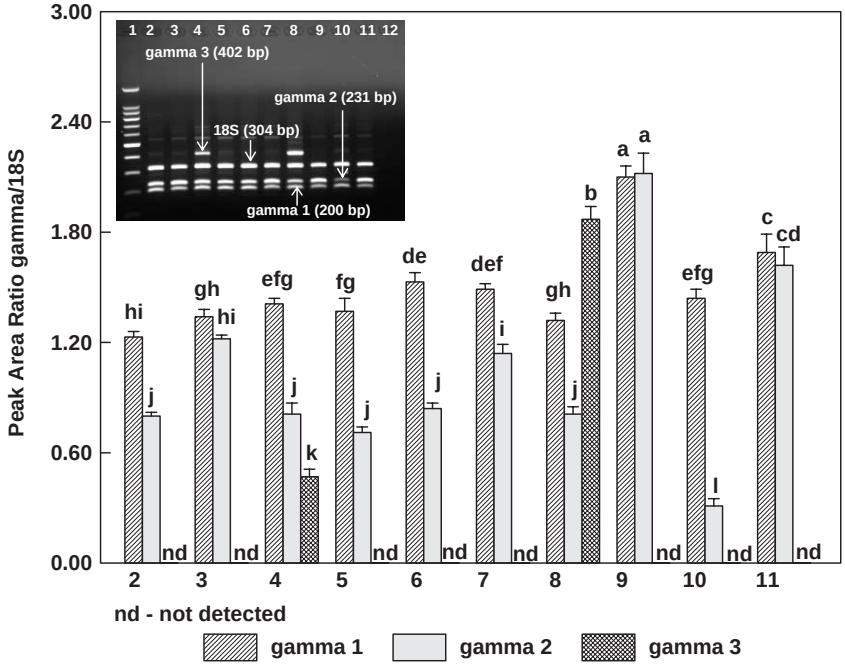

Fig. 6. Expression of AMPK gamma subunit genes in different tissues from 3 wk-old broiler chickens. RT-PCR and CE-LIF were used to quantify the level of expression relative to an 18S rRNA internal standard. Expression ratio (gamma/18S) values represent the mean±SE ($n=6$ birds). Different letters above each bar denote statistically significant ($P<0.05$) differences for mean comparisons. The inset figure presents a typical separation of PCR reactions containing AMPK gamma-1, AMPK gamma-2, AMPK gamma-3 and 18S rRNA amplicons on a 1.5% agarose gel stained with ethidium bromide. Samples are identified in the legend to Fig. 1.

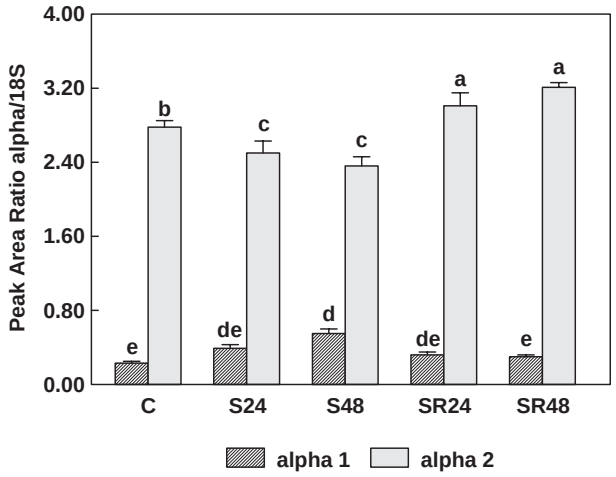

Fig. 7. Expression of AMPK alpha subunit genes in skeletal muscle tissue from 3 wk-old broiler chickens fasted for 24 h (S24) or 48 h (S48) or refed for 24 h after a prior period of fasting (SR24 and SR48, respectively). Control (C) chickens received feed ad libitum. RT-PCR and CE-LIF were used to quantify the level of expression relative to an 18S rRNA internal standard. Expression ratio (alpha/18S) values represent the mean±SE (n=6 birds). Different letters above each bar denote statistically significant (P<0.05) differences for mean comparisons.

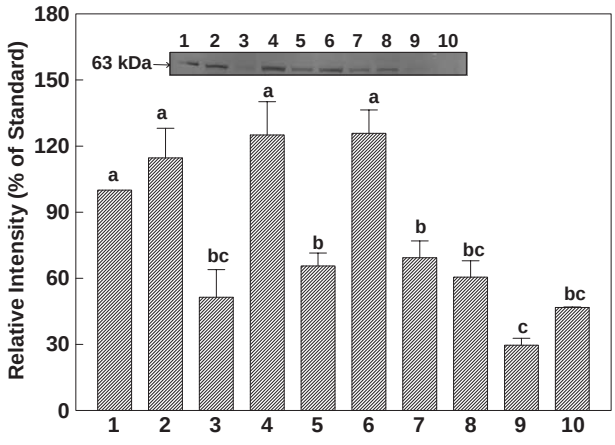

Fig. 9. Western blot analysis of the phosphorylated (active) AMPK in different tissues from 3 wk-old broiler chickens. Data are expressed as a percent of the AMPK standard calibrator (62.5 mU). Values represent the mean±SE (n=4 birds). Different letters above each bar denote statistically significant (P<0.05) differences for mean comparisons. Samples are identified as follows: (1) AMPK standard (62.5 mU), (2) liver, (3) brain, (4) heart, (5) kidney, (6) spleen, (7) duodenum, (8) skeletal muscle, (9) abdominal fat and (10) pancreas.

differ significantly from the control group, except for livers from the SR48 group where the level of phosphorylated AMPK was significantly (P<0.05) lower than in the control group.

3.5. Immunocytochemical localization of active AMPK in the hypothalamus

Since the hypothalamus plays an important role in monitoring energy balance and regulating feed intake, we were interested in studying the localization of AMPK activity to discrete hypothalamic nuclei. Therefore, we performed immunocytochemistry for phosphorylated AMPK on coronal sections of brain containing different hypothalamic nuclei. A stereotaxic atlas of the chicken brain (Kuenzel and Masson, 1988) was used to localize the hypothalamic nuclei on the immunostained sections. Lateral hypothalamus (LHy), paraventricular nucleus (PVN), periventricular nucleus (PHN), ventromedialis nucleus (VMN), infundibular nucleus (IN) and median eminence (ME) were the prominent sites of the hypothalamus that were positive for active AMPK immunostaining (Fig. 11a–f). Very strong immunostaining was observed in IN and ME (Fig. 11d–f), while LHy, PVN, PHN and VMN showed weaker immunostaining (Fig. 11a–c). Fig. 12 presents a schematic localization of phosphorylated AMPK in the chicken hypothalamus.

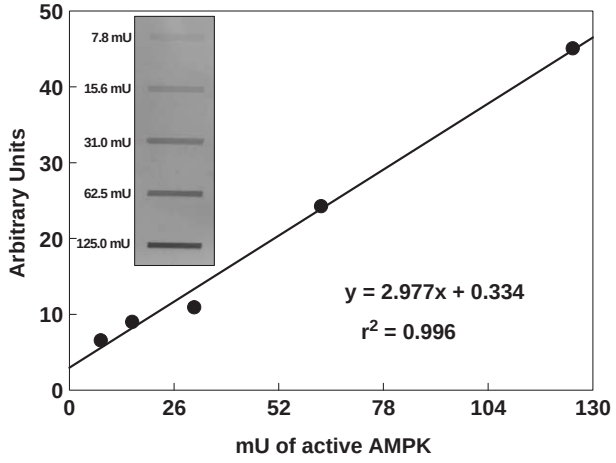

Fig. 8. Linear regression curve for the amount of active AMPK (7.8–125 mU) vs. band intensity (phosphorylation) for the quantitation of active AMPK using slot blotting. The inset figure presents a typical slot blot for different amounts of the active AMPK standard.

4. Discussion

This is the first report to identify and characterize the LKB1/AMPK pathway in chickens. Using primer-directed RT-PCR we confirmed the expression of chicken LKB1, STRAD, MO25 and AMPK subunit gene homologues. All genes, except for AMPK beta-2 and AMPK gamma-1, were mapped to individual chromosome locations. Chicken AMPK pathway gene homologues demonstrated very high homology at the amino acid level when compared to the corresponding human sequence. This indicated a high degree of conservation for these proteins.

In mammals, MO25 alpha plays a key role in stabilizing the association between LKB1 and STRAD alpha (Boudeau et al., 2003). The association between these three proteins (LKB1, STRAD alpha, MO25 alpha) increased the activity of LKB1 and enhanced the cytoplasmatic localization of the complex (Bass et al., 2003; Boudeau et al., 2003). We found that LKB1, MO25 alpha and beta and STRAD beta gene homologues were expressed in all chicken tissues examined. The alpha subunit of STRAD was expressed exclusively in brain,

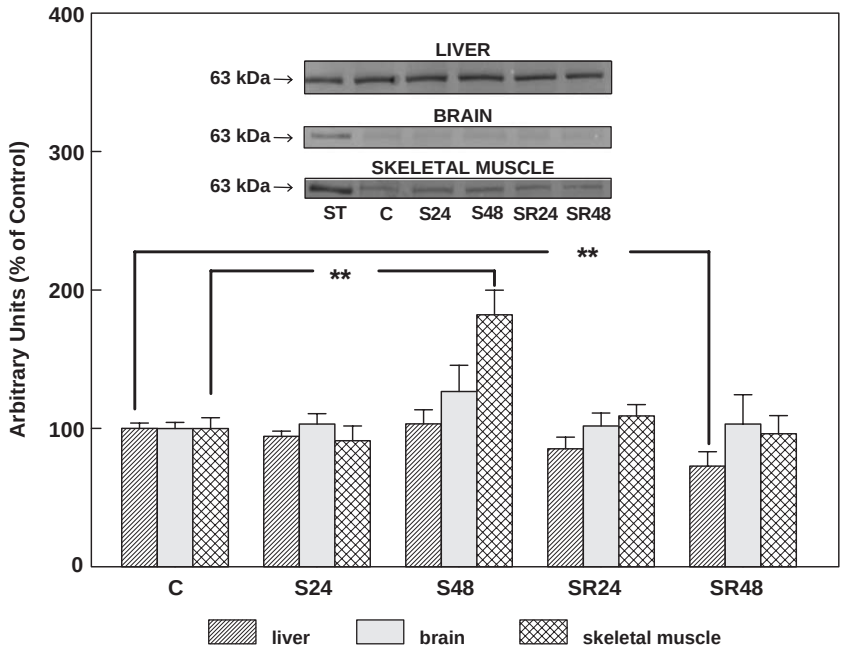

Fig. 10. Western blot analysis of phosphorylated (active) AMPK in liver, brain and skeletal muscle from 3 wk-old broiler chickens fasted for 24 h (S24) or 48 h (S48) or refed for 24 h after a prior period of fasting (SR24 and SR48, respectively). Control (C) birds received feed ad libitum. Data are expressed as a percent of the control group value for each tissue and represent the mean±SE (n=4 birds). ** Denotes statistically significant ($P<0.05$) differences for mean comparisons to the control group.

hypothalamus, heart and skeletal muscle. The highest expression of the LKB1 gene was observed in skeletal muscle and duodenum, while the lowest level was found in pancreas. Liver, kidney, spleen, duodenum, abdominal fat and pancreas in chickens showed preferential expression of STRAD beta and MO25 alpha. In brain and skeletal muscle, STRAD alpha and MO25 alpha were preferentially expressed, while in heart and hypothalamus MO25 alpha and both isoforms of STRAD were highly expressed. Our results are similar to the observations of Boudeau et al. (2003). Using Northern analysis, these authors

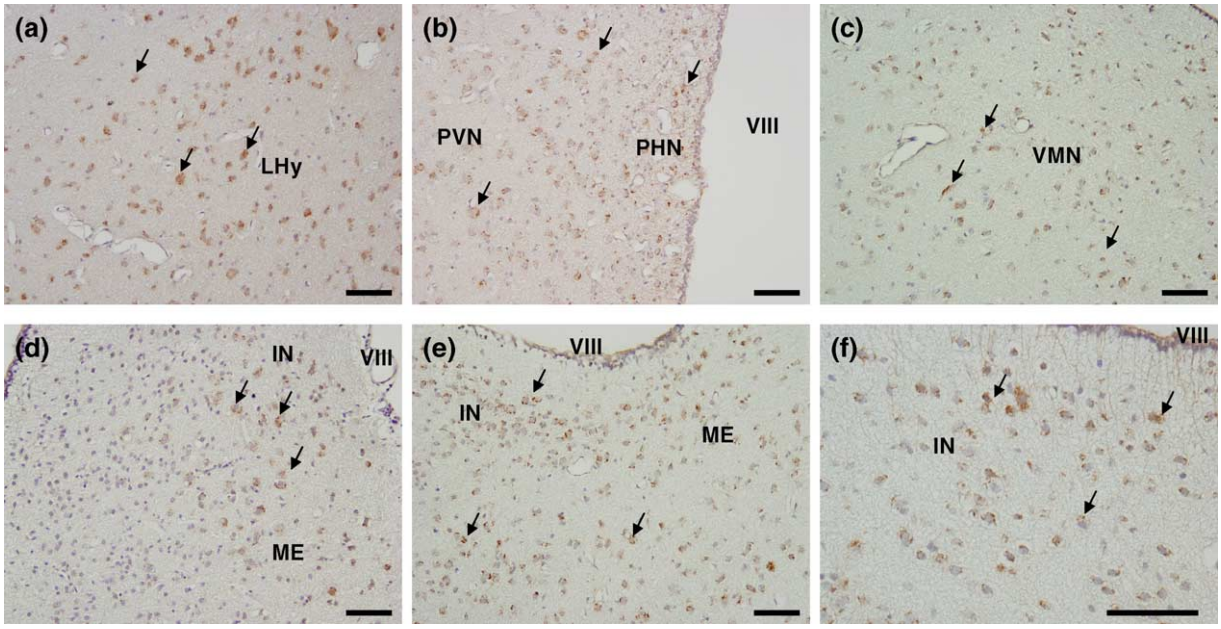

Fig. 11. Immunocytochemical localization of phosphorylated (active) AMPK in the chicken hypothalamus. Specific regions of the hypothalamus are identified as follows: (a) lateral hypothalamus (LHy); (b) paraventricular (PVN) and periventricular (PHN) nucleus; (c) ventromedial nucleus (VMN); (d–f) median eminence (ME) and infundibular nucleus (IN). (b, d–f) Third ventricle (VIII). A higher magnification (40×) of the IN stained for active AMPK is shown (f). Arrows indicate specific cells staining for active AMPK. A 50 μm scale bar is shown in each photomicrograph.

found that MO25 alpha is widely expressed in human tissues with the highest level of expression in skeletal muscle. We observed the highest expression of chicken MO25 alpha in hypothalamus. Boudeau et al. (2003) analyzed MO25 beta protein expression in different human tissues by Western blot; however, they were unable to detect corresponding mRNA using Northern blots. We found a low, but detectable, level of expression of MO25 beta mRNA in all chicken tissues examined.

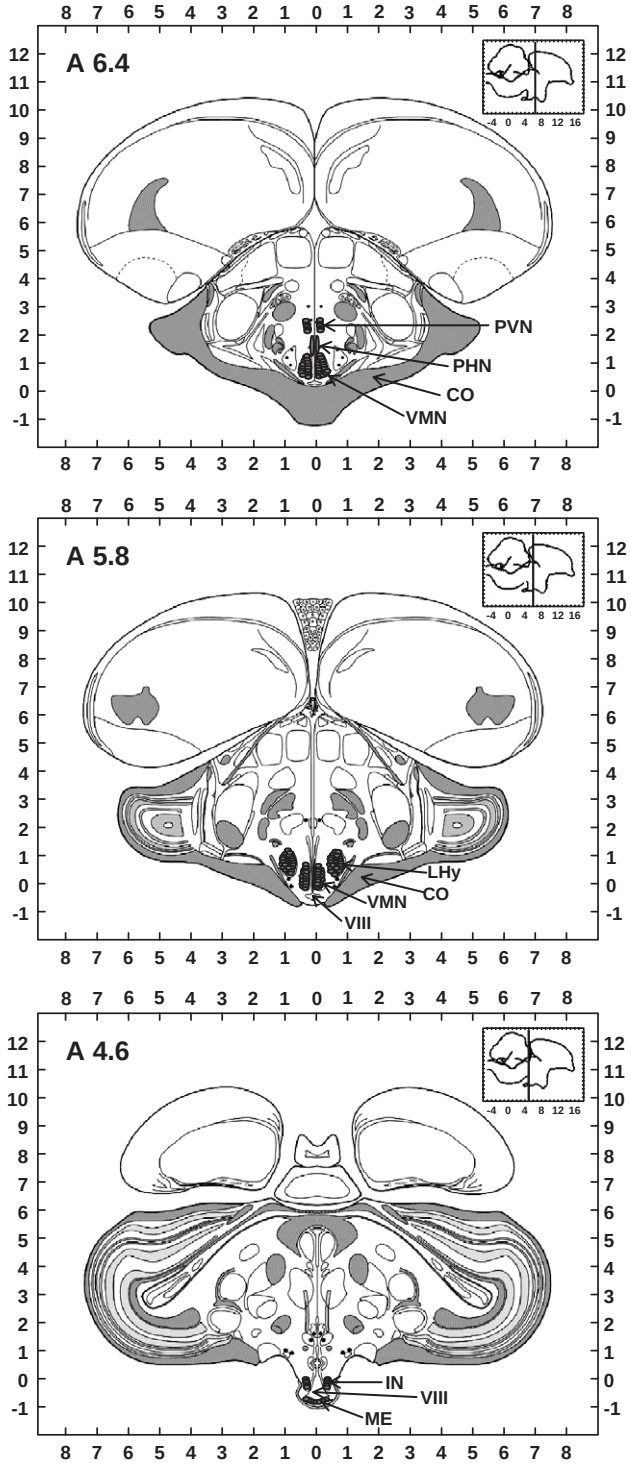

Fig. 12. Schematic localization of phosphorylated AMPK in the hypothalamus of the chicken. Black areas indicate hypothalamic regions containing the strongest immunocytochemical signal. The optic chiasma (CO) is noted and the other hypothalamic regions are identified in the legend to Fig. 11. These regions were determined according to Kuenzel and Masson (1988).

The alpha and beta isoforms of STRAD and MO25 share high homology at the amino acid level in humans and chickens. The homology for the STRAD kinase domain between alpha and beta subunits in humans and chickens was 81%; whereas, for MO25 the homology was 83% and 69%, respectively. Hawley et al. (2003) showed that the specific complex consisting of LKB1-STRAD alpha-MO25 alpha had the highest ability to enhance (over 100 fold) the activity of AMPK in HEK-293 cells. Complexes consisting of LKB1-STRAD alpha-MO25 beta, LKB1-STRAD beta-MO25 alpha and LKB1-STRAD beta-MO25 beta were less effective. Moreover, the LKB1-STRAD alpha-MO25 alpha complex enhanced the activity of 13 different kinases of the AMPK subfamily (Lizcano et al., 2004). The data obtained by Hawley et al. (2003) and our observations of a tissue-specific gene expression pattern for STRAD and MO25 in chickens suggest that in avian species the activation of AMPK may be determined by the expression of the alpha subunit of STRAD. It appears that in chickens the highest activation potential for AMPK should be expected in skeletal muscle, brain, heart and hypothalamus. This is supported by our observations that in these four tissues LKB1 and MO25 alpha were also highly expressed. Moreover, only STRAD alpha was expressed in these tissues.

Sakamoto et al. (2005) established in mice that LKB1 is a major regulator of skeletal muscle AMPK (alpha-2) activity. However, a recent study by Hurley et al. (2005) showed that AMPK could also be activated by Ca^{2+}/calmodulin dependent protein kinase kinase (CaMKK). They found that AMPK was activated much more rapidly by CaMKK beta than by the alpha isoform. To date, there has been no investigation of the role of CaMKK in the activation of AMPK in chickens, although the existence of a CaMKK alpha gene has been predicted from chicken genome sequence (GenBank Accession No. XM_415745). The identification and characterization of additional AMPK kinases (AMPKKs) that may activate AMPK in the chicken await further study.

Chicken AMPK alpha, beta and gamma subunits demonstrated very high amino acid sequence homology with corresponding human sequences, especially within their unique functional domains. This suggests a similar functional role for the AMPK heterotrimeric enzyme complex in the chicken as has been observed in mammals. The homology within the kinase domain of both alpha-1 and alpha-2 was 99% in the chicken compared to humans. The kinase domain contains a threonine residue (T-172) in the activation loop that is phosphorylated by LKB1 during activation of AMPK (Hawley et al., 1996). Within the chicken beta subunit glycogen binding domain (GBD) there was 83% (beta-1) and 95% (beta-2) homology with the corresponding human sequence. The GBD was recently characterized by Hudson et al. (2003) and they suggested that it may localize AMPK close to one of its targets, glycogen synthase, which is phosphorylated and deactivated by

AMPK in vivo and in vitro (Carling and Hardie, 1989; Wojtaszewski et al., 2002). This feature may play a key role in the repressive effect of glycogen on AMPK activation since high glycogen concentration in skeletal muscle is known to repress the activation of AMPK (Wojtaszewski et al., 2002, 2003). Furthermore, Hudson et al. (2003) suggested that binding of AMPK to glycogen via the GBD might inhibit the phosphorylation of AMPK by an upstream kinase. They also reported that the GBD is not required for binding alpha and gamma subunits in the AMPK complex, and that the alpha–gamma interacting domain alone is sufficient to bind both subunits. The amino acid sequence homology between chicken and human alpha–gamma interacting domains was approximately 95% for both the beta-1 and beta-2 subunit. Chen et al. (1999) and Thornton et al. (1998) indicated that the sequence of the alpha–gamma interacting domain of the beta-1 compared to the beta-2 subunit was nearly identical. We found that homology in the alpha–gamma interacting domain between both beta subunits of the chicken was 93%, higher than that found for humans (88%). In mammals, the three AMPK gamma subunits differ in the length of their N-terminal amino acid sequence. Gamma-2 is the largest subunit and gamma-1 is the smallest (Kemp et al., 2003). According to Scott et al. (2004), the CBS domains are the only regions of conserved sequence among gamma subunit isoforms. The homology of the CBS domain region between gamma-1, gamma-2 and gamma-3 in the chicken ranged from 53% to 96%, similar to that observed in humans (61–94%). The similarities in the amino acid sequence of the CBS domains between chicken and human gamma subunits ranged from 68% for gamma-3 to 100% for gamma-1 and gamma-2.

Studies of AMPK have shown that all three subunits, alpha, beta and gamma, are necessary to create the active enzyme complex (Dyck et al., 1996). Although the expression pattern of these subunit genes is known to vary dramatically among different tissues, the precise physiological consequences of these differences in subunit expression remain largely unknown (Stapleton et al., 1996; Thornton et al., 1998). Our results established that chicken AMPK subunit genes showed tissue-specific expression. Liver, kidney, spleen, duodenum and pancreas expressed alpha-1, beta-2 and gamma-1 subunits preferentially. Abdominal fat and hypothalamus displayed preferential expression of alpha-1, beta-2 and two isoforms of gamma (gamma-1 and gamma-2), whereas brain expressed both alpha subunit isoforms, two gamma subunit isoforms (gamma-1 and gamma-2) and the beta-2 subunit. In heart alpha-2, beta-2 and gamma-1 were expressed, while skeletal muscle predominantly expressed alpha-2, beta-2 and gamma-3 subunits. The gamma-3 subunit was expressed exclusively in heart and skeletal muscle. These findings agree with studies in mammals. For example, it has been previously noted that the alpha-1 isoform is predominantly expressed in such tissues as kidney, brain and pancreas, whereas alpha-2 is expressed preferentially in heart and skeletal muscle (Verhoeven et al., 1995; Thornton et al., 1998; Cheung et al., 2000). We have observed the same pattern for alpha subunit gene expression in chickens. Cheung et al. (2000) observed that in rat liver both alpha subunits were highly expressed. We found that in chicken liver the predominant subunit was alpha-1, whereas in brain we did not observe any differences in the expression of alpha-1 vs. alpha-2. Both alpha subunits exhibit a similar affinity for 5′-AMP, but the degree of AMPK stimulation for alpha-2 was found to be larger than that for alpha-1 (Salt et al., 1998). Moreover, differences in the subcellular localization of the alpha subunits were observed with alpha-1 localized to the cytoplasm and alpha-2 to the nucleus. Like our results in chickens, Thornton et al. (1998) observed that the human beta-1 subunit is equally expressed in all tissues, whereas the beta-2 subunit showed tissue-specific expression with a relatively high level detected in heart and skeletal muscle. In brain, the beta-2 subunit appears to be developmentally regulated in neurons and the beta-1 subunit was localized to the nucleus (Turnley et al., 1999). The beta-2 subunit in neurons and both isoforms in astrocytes were localized to the cytoplasm. Gamma-1 and gamma-2 subunit genes are widely expressed, whereas expression of the gamma-3 subunit was restricted only to skeletal muscle (Corton et al., 1995; Mahlapuu et al., 2004). It has been suggested that gamma-2 may have a specific function in the heart because a mutation in the gamma-2 gene in humans causes cardiac hypertrophy and glycogen overload (Blair et al., 2001; Daniel and Carling, 2002). Also, a mutation in the gamma-3 subunit gene is known to cause elevated skeletal muscle glycogen content in pigs (Milan et al., 2000). The chicken gamma-3 gene was expressed in heart, but its expression level was lower in comparison to other gamma subunit genes. The highest level of gamma-3 expression in chickens was observed in skeletal muscle.

Thornton et al. (1998) suggested that neither alpha-1 nor alpha-2 had a specific preference for binding either the beta-1 or beta-2 subunit during formation of the AMPK heterotrimeric complex. Moreover, Cheung et al. (2000) showed that alpha and gamma subunits could form the AMPK complex without any selective association. However, subunit isoforms interact to form different complexes in different tissues. For example, in skeletal muscle, gamma-3 preferentially forms a heterotrimeric complex with alpha-2 and beta-2 subunits (Mahlapuu et al., 2004). Based on an analysis of AMPK subunit gene expression patterns in chicken skeletal muscle it appears possible that the gamma-3 subunit would predominantly complex with the same subunits (alpha-2 and beta-2), although this will need to be confirmed in future studies using specific antibodies for immunoprecipitation of the AMPK complex.

We found that changes in energy balance caused by fasting and refeeding did not have a major impact on the expression of AMPK subunit genes in chicken liver, brain and skeletal muscle. These results indicated that AMPK expression is not regulated at the transcriptional level during changes in feeding state. This raised the question whether AMPK phosphorylation level changed during fasting and refeeding. To answer this question we determined the phosphorylation level of AMPK alpha subunits in different tissues from ad libitum fed chickens. We used an antibody that was produced against a peptide sequence corresponding to residues surrounding T-172 of human AMPK alpha-1. This was possible because the corresponding sequence in the chicken alpha subunits was identical to the human (data not shown). Significant differences

in the level of phosphorylated (active) AMPK were detected in different chicken tissues. Cheung et al. (2000) found that liver had highest level of phosphorylated AMPK. In contrast, we observed high levels of phosphorylated AMPK in liver as well as other tissues such as heart and spleen in the chicken. Our findings for the chicken agree with those of Adams et al. (2004) who reported that AMPK is largely in the inactive state in skeletal muscle at rest. In the chicken, active AMPK in skeletal muscle was approximately 50% lower that that observed in liver. Western blot analysis of chicken brain and skeletal muscle detected increases in phosphorylated AMPK during fasting. In contrast to our results, the increase in active AMPK in rat liver and brain was observed by some authors during fasting (Munday et al., 1991; Witters et al., 1994; Culmsee et al., 2001). However, Kaushik et al. (2001) and Gonzalez et al. (2004) did not find any changes in skeletal muscle during fasting. Gonzalez et al. (2004) suggested that activation of AMPK during food deprivation may be tissue-dependent and it is very likely that the hypoglycemia induced by 24 h fasting is not severe enough to effect a change in the AMP/ATP ratio leading to increased AMPK activity. We did not observe any significant changes in AMPK phosphorylation after 24 h of fasting. Therefore, it seems possible that this lack of change in active AMPK during fasting in chickens, especially in liver and brain, reflected the stability of circulating glucose levels. Plasma glucose concentration in birds is higher and apparently more resistant to food deprivation than in mammals (Hazelwood and Lorenz, 1959; Langslow et al., 1970).

The central nervous system plays an important role in energy homeostasis and feeding behavior in birds involving a complex neuronal network within the hypothalamus that regulates appetite and energy balance (Denbow, 1999; Elmquist et al., 1999; Kuenzel et al., 1999; Broberger and Hökfelt, 2001; Richards, 2003). In mammals, the hypothalamus is a major site within the central nervous system that regulates appetite (Andersson et al., 2004). Further, the arcuate nucleus (ARC), PVN, LHy and VMN are the main nuclei that play an integrative role in appetite regulation (Kuenzel et al., 1999). Not only is AMPK expressed in the brain, but within the hypothalamus AMPK expression is related to hormonal and nutritional effects on food intake and energy balance (Culmsee et al., 2001; Andersson et al., 2004; Minokoshi et al., 2004). Therefore, we investigated the presence of active AMPK in the chicken hypothalamus and its localization within specific hypothalamic nuclei. We showed positive immunostaining for phosphorylated AMPK in LHy, PVN, PHN, VMN, ME and IN of the chicken hypothalamus. The order of immunostaining intensity was found to be: IN=ME>LHy=PVN=PHN=VMN. The chicken IN is functionally equivalent to the ARC of mammals (Mikami, 1986; Kuenzel et al., 1999; Wang et al., 2001). Phosphorylated AMPK was detected in those hypothalamic nuclei thought to play a role in the regulation of appetite and energy balance in birds (Kuenzel, 1989; Snapir and Robinzon, 1989; Phillips-Singh et al., 2003). It is interesting to note that in the areas of the hypothalamus where we detected phosphorylated AMPK, previous studies have also shown expression of anorexigenic and orexigenic peptides such as neuropeptide Y, agouti-related peptide, pro-opiomelanocortin, vasoactive intestinal polypeptide, orexin (Phillips-Singh et al., 2003) and leptin (Paczoska-Eliasiewicz HE, personal communications), which function in appetite regulation and energy balance in birds (Richards, 2003). Lee et al. (2005) suggested that the increased neuronal expression of agouti-related peptide in rats during glucose deprivation is partially mediated through the activation of AMPK. We also observed very strong immunostaining for AMPK in the ME. The ME in birds is an area where connections are developed via the hypothalamo-hypohysial tracts (from the magnocellular neurosecretory cells of the supraoptic, paraventricular and preoptic regions) and the tubero-hypophysis tract (from the nucleus infundibularis) (Mikami, 1986). Further, the avian ME presents a mosaic distribution of peptide hormones and amines within neurons (Mikami, 1986).

In conclusion, we report the first experimental evidence for the expression of LKB1, STRAD, MO25 and AMPK subunit gene homologues in chickens. We observed tissue-specific expression of AMPK pathway genes. Changes in feeding state produced little significant change in AMPK subunit gene transcription. We were able to detect active AMPK via its phosphorylation state. The levels of active AMPK differed between tissues. During prolonged (48 h) fasting AMPK was activated in skeletal muscle. Active AMPK was detected in hypothalamus, especially in nuclei involved in the regulation of food intake and energy balance. Taken together, our data strongly suggest the existence of a functional LKB1/AMPK pathway in the chicken with similar characteristics to the corresponding pathway in mammals.

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